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Epidemiology of Salmonella in the Green Iguana (Iguana Iguana).

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EPIDEMIOLOGY OF *SALMONELLA* IN THE GREEN IGUANA (*IGUANA IGUANA*)

A Dissertation

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Louisiana State University and
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in partial fulfillment of the
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the Department of Pathobiological Sciences

by
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To Lorrie

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ABSTRACT

The incidence of iguana-associated salmonellosis reported in humans during the 1990's is a public health concern. This research is the first attempt to describe the epidemiology of *Salmonella* in the green iguana. Seasonal prevalence of *Salmonella* in green iguanas and their environment were estimated at a commercial iguana farm in El Salvador. The prevalence of *Salmonella* was lower in adult iguanas (44%) than in yearlings (50%) (Odds ratio (OR): 0.77, 95% Confidence Intervals (CI):0.6-0.96) and higher in male iguanas (50%) than females (41%) (OR: 1.5, 95% CI: 1.2-1.8). *Salmonella* recovery was lower in the fall (41%) sampling period than in the spring (49%) (OR: 0.7, 95% CI: 0.6-0.9). Recovery of *Salmonella* from the ovary of one (4%, n=25) female iguana, 5 (14%, n=35) hatchling yolk-sacs, 3 (5%, n=66) embryonic yolk-sacs, and one (2%, n=66) embryo suggests that this organism may be transmitted vertically. *Salmonella* was not isolated from feed samples, food preparers' hands, insects, or well water. *Salmonella* was isolated from the soil (44%, n=340) and the water basins (37%, n=116) within the pens housing iguanas.

Bayesian estimates of the sensitivity and specificity of the polymerase chain reaction (PCR) assay, enzyme-linked immunosorbent assay (ELISA), and microbiological culture protocol used for *Salmonella* detection in the iguanas in this study were 0.93 and 0.95, 0.81 and 0.86, and 0.67 and 0.99 respectively.

A series of intervention studies were performed to create a *Salmonella* clearance and infection model to evaluate a commercial *Salmonella* vaccine. Young iguanas treated with 10 mg/kg enrofloxacin suspension *per os* were less likely to be *Salmonella*

positive than untreated iguanas (χ^2=36.2, 1 df, p<0.0001). Iguanas cleared with enrofloxacin and re-infected with *Salmonella* Typhimurium strain 524 were more likely to be *Salmonella* positive than uninfected iguanas (χ^2= 13.3, 1 df, p<0.001). Latent infection was observed in six (32%) cleared, but uninfected, control iguanas. Vaccination with a commercially licensed avirulent attenuated *Salmonella* Typhimurium did not protect vaccinated iguanas against re-infection with *Salmonella* Typhimurium strain 524 (χ^2= 0.06, 1 df, p=0.8).

CHAPTER 1

INTRODUCTION

Reptiles constitute the fastest growing sector of the pet market in the United States of America. A 1994 National Pet Owner's Survey recorded over 7.3 million pet reptiles in the USA. United States Fish and Wildlife Service statistics confirm that the green iguana is becoming an increasingly popular pet as evidenced by the importation of over 640,000 immature animals in 1997.

Captive propagation of green iguanas occurs primarily in Central and South America with the largest proportion of hatchlings shipped to the USA. Commercial breeding stock is maintained in open earthfloor pens. Male and female iguanas are housed together from November to January for the mating season. Eggs are deposited in soft soil between January and March in 60 to 80-cm-deep nests. The adults are then removed from the pens to prevent damage to the nests. Incubation time and hatching are influenced by environmental conditions including soil moisture and temperature, but the hatching season begins in late March and continues through June. Mature iguanas and hatchlings are fed a protein concentrate supplemented with a variety of vegetables and fruits (depending on seasonal availability). The hatchlings are held in the breeding pen for a minimum of 7 days until consigned to wholesalers.

Salmonella infection was first identified in snakes in 1944 (McNeil and Hinshaw, 1945), and in turtles and lizards in 1946 (Hinshaw and McNeil, 1946). Until the 1960's, reports of reptile-associated salmonellosis were rare. During the 1970's, approximately 4% of US households owned pet turtles and these animals accounted for

14% (280,000) of all reported cases of salmonellosis in children under ten years of age in the USA (Gangarosa, 1985). In 1975, the US Food and Drug Agency implemented an interstate ban on commerce in turtles. This effectively halted the sale and ownership of turtles within the continental US and markedly reduced the number of turtle-associated cases of salmonellosis (Cohen et al., 1980).

Recently, reports of salmonellosis from non-turtle reptile reservoirs have gained national attention (CDC, 1992a; CDC, 1992b; Ackman et al., 1995). In most documented cases, the strain of *Salmonella* isolated from the patient was common to a pet reptile, confirming the source of infection. The US Center for Disease Control and Prevention has estimated that in 1996 there were over 50,000 cases of reptile-associated salmonellosis (Meehan, 1996).

The increased popularity of the green iguana, with attendant risks of salmonellosis in owners and contacts of both clinically affected and normal green iguanas, merits study. There have been no documented epidemiological investigations to demonstrate when and how the commercial green iguana becomes infected with *Salmonella*. Specifically, objectives of this research are to:

1. Describe the prevalence distribution of *Salmonella* infection in green iguanas from a commercial breeding facility exporting hatchlings to the USA. A specific aspect relates to the occurrence of vertical and horizontal transmission of infection.

2. Estimate the sensitivity and specificity of microbiological culture, enzyme-linked immunosorbent assay, and polymerase chain reaction for detecting *Salmonella* in green iguanas (*Iguana iguana*).
3. Create a model for *Salmonella* clearance in green iguanas by administering enrofloxacin.
4. Create a model for experimental *Salmonella* infection in green iguanas after clearing them with enrofloxacin.
5. Evaluate the efficacy of an avirulent live attenuated vaccine against *Salmonella* colonization in green iguanas.

CHAPTER 2

LITERATURE REVIEW

2.1 Biology of the Green Iguana

Green iguanas are members of the class Reptilia, order Squamata, suborder Sauria, infraorder Iguania and family Iguanidae (Barten, 1996). The family Iguanidae is the dominant family of lizards in the New World and is characterized by pleurodont dentition. The green iguana was first described by Linnaeus in 1758 and classified as *Lacerta iguana* (Etheridge, 1982). In 1934, Dunn re-classified the green iguana using the currently accepted scientific name (*Iguana iguana iguana*).

The green iguana is widely distributed from northern Mexico and southward through Central and South America to Paraguay and southeastern Brazil (Etheridge, 1982). The green iguana also inhabits several island chains, including the Lesser Antilles (Etheridge, 1982). The iguana is adaptable and has increased its range to include rural inhabited dwellings.

Green iguanas are one of the largest extant herbivorous lizards. Body size in green iguanas can be quite variable, with adult snout to vent length ranging from 26-45 cm (Dugan, 1982). Body weight is also variable, ranging from 0.9-3.87 kg (Dugan, 1982). In a group of Panamanian green iguanas, the mean female weight was 74% of the mean male weight (Dugan, 1982).

Green iguanas are sexually dimorphic. In addition to their larger size, males have larger heads, longer spines on their dorsal crest, and larger femoral pores. The development of secondary sexual characteristics in male green iguanas is slow and

coincides with reproductive maturity. Male green iguanas undergo a color change with maturity and also during the breeding season. Mature male green iguanas are a dull gray, gold or tan, but during the breeding season their color intensifies to a bright gold to red-orange (Dugan, 1982). Sexual maturity in male and female green iguanas has been reported in animals greater than 27 cm snout to vent length (Dugan, 1982). The age to sexual maturity may vary among geographical regions depending upon climate and diet, but occurs during the third year of life, at approximately 30 months.

Iguanas are oviparous, laying a single annual clutch of 24-72 eggs depending on maternal size and nutritional state (Rand, 1968). The female iguana may travel up to 3 km to find a suitable nest site (Montgomery et al., 1973). The eggs are deposited in soft soil between January and March, with hatchlings emerging in April through June when food is abundant (Rand, 1972).

Green iguanas are ectothermic and depend upon their environmental temperature to regulate their core body temperature. Metabolic rate, immune function, and reproductive cycle are directly related to core body temperature. Green iguanas routinely bask during the early morning hours and use their large surface area as a heat trap. Reptiles are capable of inducing hyperthermia by modifying behavior and basking patterns (Avery, 1982).

Green iguanas are one of the few lizards that remain herbivorous throughout their life (Rand, 1978). The diet of these arboreal lizards includes a variety of leaves, blossoms and fruits (Rand et al., 1990). The gastrointestinal tract of the green iguana consists of an esophagus, monogastric stomach, short small intestine, large partitioned

colon, rectum, and cloaca. The partitioned colon is the location of hindgut fermentation. The functional significance of the partitioned colon is not well understood, however, it has been suggested that it slows digest passage time and increases the absorptive surface area of the colon (Iverson, 1982). Mammalian herbivores feed constantly to satisfy caloric requirements. In contrast, the green iguana spends less than 10% of its active day feeding because it requires a short time to fill the gastrointestinal tract (Moberly, 1968).

Herbivorous mammals derive a portion of their caloric requirements from the fermentative activity of protozoa and bacteria colonizing the gastrointestinal tract. These microbes are responsible for the degradation of cellulose and components of vegetation into usable volatile fatty acids that can be incorporated into the Kreb's cycle. The domestic rabbit (*Oryctolagus cuniculus*) derives 4-12% of its caloric needs from the microbial processes in the hindgut (Bailey and McBee, 1964), whereas the beaver (*Castor canadensis*) derives approximately 19% of its maintenance energy from the hindgut (Hoover and Clarke, 1972). The green iguana obtains a greater proportion of its daily caloric requirements, approximately 30%, from the activity of the microbial flora of the hindgut (McBee and McBee, 1982). Unlike mammals, reptiles must bask in radiant heat to maintain a core temperature to maximize fermentation in the hindgut. The primary volatile fatty acids produced in the colon of the green iguana include acetic, propionic and butyric acids (McBee and McBee, 1982). The production of acetic acid in the hindgut of the green iguana is consistent with mammalian endotherms. The reason for the high production of butyric acid, however, is not apparent (McBee and McBee, 1982).

The gastrointestinal microflora responsible for hindgut fermentation in the green iguana has not been defined, although an unidentified complex of bacteria with *Lampropedia merismopedioides* as the principal species has been isolated from the colon of wild-caught individuals (Troyer, 1982). This organism has also been isolated from the rumen of cattle and sheep (Hungate, 1966). Another microbe, an undescribed large ciliated protozoan resembling the holotrichs of the rumen, has also been routinely identified in wild green iguana hatchlings (Troyer, 1982). *Clostridium* and *Leuconostoc* have also been predominant species identified (McBee and McBee, 1982) in green iguanas, and these microbes, as well as those yet undescribed, are considered important commensals in the digestion of plant products.

The transfer of the hindgut microbial flora in neonatal mammals occurs through close contact with the mother or fecal contents of other older conspecifics. The passage of the hindgut microbial flora in green iguanas does not occur through close contact because the female green iguana leaves the nesting grounds once the eggs are deposited (Rand, 1968). When the hatchlings emerge during the spring (April-June) they inhabit different niches than adults. In areas where the adults and hatchlings do share a similar range, the adults are often located in the high canopy (15-30m) and the hatchlings in the low vegetation (1-5m) (Troyer, 1982).

Hatchling green iguanas can consume a significant amount of soil within their nest and also lick eggshells, or each other (Troyer, 1982). Although this might be a potential source of microbial passage, a study evaluating the colonic contents of hatchling green iguanas for *L. merismopedioides* and the ciliated holotrich protozoan

found that animals exposed to the nest soil and eggshells were no more likely to be transfaunated with those microbes than captive hatched animals (Troyer, 1982). In the same study, there was no detectable fecal material in the nests. A comparison of the nest soil and surface soil for total microbe content was also unremarkable and suggested that maternal contamination of the nest was not a likely source of the organisms, although study results were limited by the total number of animals sampled (Troyer, 1982). The only technique found to consistently reproduce the microbial flora in hatchlings was reported when hatchlings were offered fresh adult fecal material or if the animals were housed with an older hatchling with a complete microflora (Troyer, 1982).

2.2 Commercialization of the Green Iguana

Reptiles constitute the fastest growing sector of the pet market in the United States of America. A 1994 National Pet Owner's Survey recorded over 7.3 million pet reptiles in the USA. United States Fish and Wildlife Service statistics confirm that the green iguana is becoming an increasingly popular pet as evidenced by the importation of over 640,000 immature animals in 1997. The popularity of the green iguana is cosmopolitan, as the green iguana is the second most traded vertebrate species in world commerce according to the World Conservation Monitoring Center (Rodda, 1993). The popularity of this species created a niche for captive propagation programs.

Captive propagation of green iguanas occurs primarily in Central and South America, with the largest numbers shipped to the USA. Green iguanas are protected under the Convention on International Trade of Endangered Species (CITES) and are a

appendix II animal. Permits and farm inspections are managed by the Department of Agriculture of the host country.

Commercial breeding stock may be acquired through purchase or capture of wild specimens. The commercial breeding stock and hatchlings are maintained in open earthfloor oval pens constructed of tin. Shelters constructed from bamboo or concrete cinder blocks provide protection from extreme weather. The specific diet offered to captive green iguanas varies depending upon the location of the farm, but will include a protein concentrate supplemented with a variety of vegetables and fruits (depending on seasonal availability). Non-chlorinated, well water is offered as drinking water.

Male and female breeding stock are housed separately from February to October. During November, animals are transferred from single gender pens and placed into breeding pens at a ratio of 1 male: 3 females. Males are removed from the pens after copulation has occurred during January. Eggs are deposited in soft soil between January and March in 60 to 80-cm-deep nests. The female iguanas are then removed from the pens to prevent damage to the nests. Incubation time and hatching are influenced by environmental conditions including soil moisture and temperature, but the hatching season begins in late March and continues through June.

Hatchlings remain in the large breeder pens until they are collected for export or until October when breeding commences and are offered the same diet as the adult animals. The iguana eggs hatch over a 2-month period, therefore the cohort may vary in age by as much as 60 days. The animals are only handled to be collected for export and are transported in a mesh bags. Shipments to the US require that no more than 15

animals be placed in an individual bag and that the transport box meets the minimum requirements. Food and water are withheld during transport.

2.3 *Salmonella*

Salmonella are Gram-negative, usually motile, facultative anaerobes that conform to the definition of the family Enterobacteriaceae. *Salmonella* have a cosmopolitan distribution (Acha and Szyfres, 1987). Most, if not all, of the described species are considered pathogenic (Smith, 1991). *Salmonella* are typically aerogenic, although there are some exceptions (*S.* Dublin) (LeMinor, 1984). Hydrogen sulfide production is another hallmark of this group, although several strains of *S.* Paratyphi and *S.* Cholerasuis do not produce it (LeMinor, 1984). Citrate is typically the sole carbon source. *Salmonella* Arizonae, which are routinely isolated from reptiles, use malonate as a primary carbon source (LeMinor, 1984). *Salmonella* are typically non-lactose fermenters, although *S.* Arizonae routinely ferment this sugar (LeMinor, 1984).

The genetic map of *S.* Typhimurium (Sanderson and Hartman, 1978), differs little from *E. coli* K12 (Bachmann and Low, 1980) with an overall DNA-DNA homology approximating 90% (Sayers and Whitt, 1994). Chromosomal transfer is possible from *E.coli* to *Salmonella*, *Salmonella* to *E. coli*, and from *Salmonella* serotype to *Salmonella* serotype through conjugation. The acquisition of plasmids that confer antibacterial resistance or biochemical characteristics are frequently transferred by this mechanism (LeMinor, 1984). The DNA homology shared by organisms within the family Enterobacteriaceae has led to confusion among microbiologists regarding taxonomy. The current classification of the genus *Salmonella* includes two species: *S.*

enterica and *S. bongori* and six subspecies of *S. enterica* including *enterica* (subspecies I), *salamae* (subspecies II), *arizonae* (subspecies III), *diarizonae* (subspecies IIIb), *houtenae* (subspecies IV), and *indica* (subspecies V) (Popoff and LeMinor, 1997). Subspecies I is routinely isolated from humans, whereas the other subspecies are frequently isolated from poikilotherms and the environment. Serotype identification for subspecies I are designated by the geographical location from which the serotype was initially isolated (Popoff and LeMinor, 1997). The serotypes of the remaining subspecies are designated by their antigenic formula. There are currently 2,435 described *Salmonella* serotypes and the majority are classified under subspecies I (1,435) (Popoff and LeMinor, 1997). The *arizonae* and *diarizonae* subspecies have 94 and 321 serotypes respectively.

Salmonella are serotyped according to their O (heat stabile somatic) antigens, Vi (heat labile capsular) antigen, and H (flagellar) antigens (McWhorter-Murlin and Hickman-Brenner, 1994). The Kauffman-White scheme is used to list the antigenic formulae which are expressed as: O antigen(s), Vi antigens (when present): H antigen(s) (phase 1): H antigen(s) (phase 2, when present)(McWhorter-Murlin and Hickman-Brenner, 1994). Organisms with O antigens in common are placed into similar O groups and arranged alphabetically by H antigens. The specificity of the O factors is determined by the composition of the polysaccharide and may be altered by mutation or bacteriophage conversions (Stocker and Makela, 1971).

Lysogenization by phages may change the O antigen formulae for an organism. Phages can be differentiated from one another serologically (LeMinor, 1968) and the

factors associated with phage conversion are underlined when using the Kaufmann-White scheme. Currently, phage typing is limited to a few serovars, including *S.* Typhi, *S.* Typhimurium, *S.* Dublin, *S.* Enteritidis, *S.* Heidelberg, and *S.* Schottmuelleri (Clarke and Gyles, 1993). Identification of these phage types may be important in epidemiologic studies. Antibiotic resistance patterns, biotyping, and plasmid profile analysis are other diagnostic techniques that may be used to identify a *Salmonella* organism beyond the serovar. These techniques are routinely employed in epidemiologic studies to determine the source of a pathogen in a disease outbreak.

2.4 *Salmonella* Pathogenesis: Virulence Factors

Infection in animals and humans with *Salmonella* may result in serious disease or give rise to a reservoir for other species and contacts within that environment. The interaction of *Salmonella* with a host gives rise to a number of clinical presentations including; inapparent infection, recovered carrier state, enteritis, septicemia and combinations of disease syndromes (Clarke and Gyles, 1993).

A number of virulence factors have been identified in *Salmonella* that enable the organism to invade and infect a host. The majority of *Salmonella* are motile, using a flagella to contact enterocytes (Finlay and Falkow, 1989). Flagella may be organized into a bundle at one pole, creating a smooth swimming activity, or project from the organism, resulting in a tumbling pattern. *Salmonella* Typhimurium with a single polar flagella is more invasive than those that project from the organism (Jones et al., 1992). Virulence factors, such as flagella, are the basis of immunological diagnostic tests which offer rapid identification after enrichment culture. An enzyme linked

immunosorbent assay (Salmonella ELISA 96/1) offered by Bioline (Vejile, Denmark) uses affinity purified rabbit antibody specific to *Salmonella* flagellar antigens.

Siderophores are iron scavenging chelators produced by bacteria when iron concentrations are low within a host. Bacteria excrete siderophores into the host tissues to chelate iron, which are incorporated into the bacteria through specific outer-membrane receptor proteins produced in response to a low concentration of iron (Finkelstein et al., 1983). The exact role for scavenging systems in an intracellular pathogen is under debate. *Salmonella* Typhimurium produces a phenolate siderophore (enterochelin) considered to be a virulence factor. However, Benjamin et al. (1985) demonstrated that the siderophore was not essential for virulence.

Lipopolysaccharide (LPS) is a major determinant of virulence in *Salmonella.* The LPS is composed of an internal Lipid A embedded in the outer membrane core region of an antigenic O region. (Clarke and Gyles, 1993). Organisms that lack the O core region are classified as "rough" mutants and are less virulent than organisms with an intact O core region ("smooth"). The lack of the O core region results in an increased susceptibility of the rough mutant. The O core region protects the organism by increasing the distance between the cell membrane and complement-mediated mechanisms. The chemical composition of the O antigen is also an important consideration in activating complement by the alternate pathway, and may affect the rate of phagocytosis by macrophages (Saxen et al., 1987). The endotoxic properties of LPS also contribute to virulence. Bacterial LPS is capable of stimulating a cascade of inflammatory mediators and immunoregulatory cytokines, leading to vascular damage

and thrombosis (Clarke, 1985). Many of the clinical signs associated with *Salmonella* infection in humans, including cramps, and fever, are attributed to LPS.

Many enteric pathogens rely on invasin genes to penetrate host enterocytes. Invasin genes are believed to mediate an extensive actin rearrangement in the host cell, resulting in a distortion of the cell membrane, enabling the organism to invade. Disruption of the invasion A gene in a strain of *Salmonella* Typhimurium prevents the organism from invading enterocytes (Altmeyer et al., 1992). The invasion genes (A-H) are highly conserved among *Salmonella* (Sayers and Whitt, 1994). The conservation of these genes across the genus has resulted in the development of improved molecular techniques (PCR) to diagnose *Salmonella* infection (Cohen et al., 1996).

2.5 Isolation of *Salmonella*

2.5.1 Standard Microbiological Methods

Microbiological culture is conventionally used to detect *Salmonella* in various tissues and excretions (Smith, 1991). The standard microbiological method incorporates a highly selective enrichment broth that inhibits genera other than *Salmonella*. Pre-enrichment media, containing lactose, are generally used to provide additional energy to injured bacteria and increase the probability of recovery. However, Siebling et al. (1975) reported that pre-enrichment with lactose broth before enrichment in tetrathionate broth reduced the recovery of *Salmonella* from turtles. The four most commonly recommended enrichment medias for *Salmonella* are tetrathionate broth with or without brilliant green, modified semisolid Rappaport-Vassiliadis, and Selenite broth. Enrichment media should be selected based on the subspecies of *Salmonella* to be

isolated. Tetrathionate may inhibit the multiplication of certain *Salmonella* serotypes if the inoculum is small (Van Schothorst et al., 1977). Selenite is toxic to *Salmonella* Cholerasuis (Smith, 1952). Rappaport enrichment broth has been used to consistently isolate different subgenera of *Salmonella*, however only 27% (3/11) of the strains of subgenus III were subcultured from Rappaport (Vassiliadis, 1968). Samples are routinely incubated under aerobic conditions at 37°C in the enrichment media for 18-24 hours. After enrichment, the sample is plated onto a selective medium for isolation. MacConkey agar and eosin methylene blue are of low selectivity. Media of intermediate selectivity include xylose-lysine-desoxycholate (XLD) agar and *Salmonella-Shigella* agar. Bismuth sulfite agar and xylose-lysine tergitol 4 (XLT-4) are considered highly selective. Bismuth sulfite, XLD, and XLT-4 contain hydrogen sulfide (H_2S) indicator systems that can detect lactose-fermenting *Salmonella*.

Suspect colonies may be inoculated onto a selective screening media such as lysine iron agar (LIA) and triple iron agar (TSI). Lysine iron agar is used because *Salmonella* decarboxylate lysine and produce H_2S. On the TSI media, *Salmonella* ferment glucose, produce gas and hydrogen sulfide. There are other biochemical tests or a slide agglutination test with antisera for *Salmonella* O groups that may be used to confirm *Salmonella* spp. (Guthrie, 1992).

Delayed secondary enrichment (DSE) is a technique used in the poultry industry to increase recovery rates of *Salmonella* in diagnostic and environmental samples. Delayed secondary enrichment may be beneficial to *Salmonella* organisms that are damaged by antibiotics, require additional time for multiplication because of low

numbers, or if competing bacteria are present in the sample. A sample enrichment period of 24 h is used prior to *Salmonella* isolation on selective media. In a questionnaire administered to veterinary and independent diagnostic laboratories in the United States, 51% of respondents confirmed the use of a 24 hour enrichment period for culturing *Salmonella* (Waltman and Mallinson, unpublished data). Waltman et al. (1991) collected 4,377 samples from poultry, including yolk-sacs from 1-day old chicks, tissues from Pullorum-typhoid reactors, and environmental swabs, to evaluate two different selective medias and the recovery rates associated with a 24 h enrichment period and a 5-day DSE. Samples were initially inoculated into tetrathionate broth and incubated for 24 h at 37° C. Samples were plated onto brilliant green agar or brilliant green agar with novobiocin and incubated for 24 h at 37°C. The remaining tetrathionate broth was retained at room temperature for five days. After this period, 1.0-ml of the suspension was added to a fresh tube of tetrathionate broth and incubated for 24 h at 37°C. An aliquot was then collected from the sample and plated onto brilliant green agar with novobiocin and incubated for 24 h at 37°C. Presumptive *Salmonella* colonies were characterized with biochemical tests. *Salmonella* were isolated from 464 samples (11%). Two-hundred and sixty-nine (58%) *Salmonella* were isolated after the 24 h enrichment and 421 were isolated after the 5 day DSE. Forty-three (9%) of the *Salmonella* were isolated after the 24 h enrichment and not the DSE, compared to 195 that were only isolated after the DSE. The DSE improved overall recovery of *Salmonella* by approximately 64%. Waltman et al. (1991) also evaluated a 3-day DSE

and found that the combination of a 24 hour and 5-day DSE provided the most optimal results.

Although microbiological culture is considered to be the standard in clinical diagnostic testing, the true test sensitivity and specificity are unknown. The reliability of culture may be affected by several factors, including method used to collect the sample, quantity of sample collected and submitted, temporal and seasonal variation in shedding and the method of culture (Smith, 1991; Hird et al., 1984; Owens et al., 1983).

2.5.2 Isolation of *Salmonella* from Reptiles

The microbiological techniques used to isolate *Salmonella* from reptiles are based on those developed for *Salmonella* subspecies from endothermic animals. *Salmonella* isolates from reptiles are commonly classified in subspecies III and IIIb and may utilize different biochemical pathways than other subspecies. Comparative studies to evaluate isolation success for reptile *Salmonella* serotypes using available enrichment broths and selective media are limited (Harvey and Price, 1983; Kodjo et al., 1997).

A study was performed at the Bristol Zoological Gardens to evaluate isolation techniques using the fecal material from twenty different reptiles species (Harvey and Price, 1983). Approximately 2 g of feces was divided equally and placed into either selenite F enrichment broth or Muller-Kauffmann tetrathionate broth followed by incubation under aerobic conditions at 43°C for 24 hours. A sample from each of the enrichment broths was subcultured onto three selective agars, including brilliant green MacConkey agar, desoxycholate citrate agar, and de Loureiro's three stock solution modification of bismuth sulfite agar, and incubated at 37°C for 24 hours. *Salmonella*

suspect colonies were further evaluated with appropriate biochemical tests, and 16 different *Salmonella* serotypes were identified. *Salmonella* were more consistently isolated from selenite F (81%) than Muller-Kauffmann tetrathionate (63%). These findings suggest that selenite F would be the preferred enrichment broth for reptile serotypes, however, the sample size in this study was limited (N=20). The selectivity of an enrichment broth may restrict the multiplication of certain *Salmonella* serotypes. When the results of the two enrichment broths were combined in this study, the number of isolates increased by 38%. Reptiles may harbor multiple serotypes and the use of multiple enrichment broths may increase the recovery rate of salmonellae. Delayed culture may also increase the recovery rate of certain *Salmonella* serotypes. Subculture of selenite F and Muller-Kaufmann tetrathionate at 24 h and 48h increased the chances of recovering multiple serotypes (Harvey and Price, 1983).

Kodjo et al. (1997) investigated the use of two different enrichment broths with three selective agars. Fecal samples were collected from a population of 32 chelonians comprising 9 species and assayed for *Salmonella*. Feces were enriched in either selenite or tetrathionate and incubated at 37°C for 24h. A sample was collected from each of the enrichment broths and subcultured on to either Rambach, Salmonella-Shigella, or XLT-4 agar and incubated for 37°C for 24 h. *Salmonella* suspect colonies were further characterized with various biochemical tests, and 13 of the 32 samples were *Salmonella*-positive. Thirteen isolates were recovered from selenite broth, compared to eleven with tetrathionate. All thirteen isolates grew on Rambach and *Salmonella-Shigella* agar, compared to twelve on XLT-4.

The method of specimen collection can affect *Salmonella* recovery rate. Wells et al. (1974) compared three methods for isolating *Salmonella* from turtles: sampling contaminated environmental water, whole carcasses, and individual organs. Fifty turtles were used to evaluate each isolation method. Turtles were housed individually to reduce the likelihood of cross transmission of salmonellae. A sample of water was collected from the container of each unfed turtle one week after being enrolled in the study. After water samples were collected, animals were humanely euthanized and samples were collected from the yolk, bile, liver, spleen, small intestine, colon, and kidney. The second group of turtles were individually blended and a representative sample incubated at 37°C for 48h. All culture samples were enriched in tetrathionate broth containing brilliant green broth and incubated at 37°C for 48h . A sample from the enrichment broth was streaked on to brilliant green agar and incubated at 37°C for 48h. *Salmonella* was isolated from 17 animals with the blender method, and from 24 water samples. The difference between the two methods was not statistically significant. *Salmonella* was recovered from bile (N=1), liver (N=1), small intestine (N=10), colon (N=10), and kidney (N=1). The overall frequency of recovery from individual parts was significantly lower than the excretion or blending technique, and collecting multiple samples may increase the probability of recovering *Salmonella* from a reptile.

2.5.3 Non-Culture Diagnostic Tests

Historically, microbiological culture has been used to detect *Salmonella* from various tissues and excretions (Smith, 1991). Although culture has been considered the gold standard for detecting *Salmonella*, test characteristics, including sensitivity and

specificity, are unknown. There are a number of parameters that can affect the reliability of culture, including method of specimen collection, quantity or type of sample collected, temporal or seasonal variation in shedding, and method of culture (Smith, 1991; Hird et al., 1984; and Owens et al., 1983). An additional limitation of microbiologic culture is the time required to confirm a diagnosis, which may exceed 48 h to isolate *Salmonella* with microbiologic culture, and 96 h with biochemical tests (Cohen et al., 1994). The delay imposed by culture techniques may defer initiation of appropriate antimicrobial therapy and control procedures. Culture of certain microbes can be difficult, if not impossible, based on our current knowledge of growth requirements. The inability to confirm the presence of a fastidious organism can influence the management of a clinical case. Advances in diagnostic testing have led to the introduction of new technologies, including enzyme-linked immunosorbent assay and polymerase chain reaction assays. These diagnostic tests offer rapid diagnosis and are also both sensitive and specific.

The impact of *Salmonella* on human and animal health has created a need for rapid and accurate detection methods for *Salmonella* in animal and environmental samples. Enzyme-linked immunosorbent assays combine a specific anti-immunoglobulin with an enzyme to detect a specific microbial antigen. The ELISA procedure has been used extensively in the food industry to detect *Salmonella* (Ibrahaim, 1986; Todd et al., 1987; Flowers et al., 1989 Van Poucke, 1990). Limited testing has indicated potential benefits of the ELISA in characterizing the *Salmonella*

status of animal and environmental samples (Desmidt et al., 1994; Pelton et al., 1994; Tan et al., 1997).

Desmidt et al. (1994) compared a commercial ELISA (*Salmonella*-Tek, Organon Teknika, Co.) to microbiological culture for detection of *Salmonella* from cloacal swabs of poultry and drag-swabs of litter. Out of the 73 cloacal samples collected from chickens, 22 (30%) were positive (ELISA) and 25 (33%) cultures yielded a *Salmonella* isolate. Out of 42 litter samples, 39 (88%) were positive (ELISA) and 22 (48%) cultures yielded a *Salmonella* isolate. There was no difference between the two detection methods when comparing cloacal swabs, however the ELISA was more sensitive than culture for detecting *Salmonella* in litter samples. Poultry litter with a low pH or water activity is bactericidal to salmonellae. The high sensitivity of the ELISA was attributed to the detection of antigen from dead *Salmonella* cells.

Pelton et al. (1994) compared an antigen capture ELISA to microbiologic culture for detection of *Salmonella* from equine, avian, and reptile fecal samples. Thirty-five culture negative samples and 35 culture positive samples were assayed with the ELISA. Of the culture-negative samples, one (3%) was positive with the ELISA in contrast to the culture-positive samples, of which eleven (31%) were positive with the ELISA. Test sensitivity was 69% and test specificity was 97%. The ELISA used in this study would be an acceptable indicator of positive status, but only a fair indicator of negative status.

Tan et al. (1997) compared a lipopolysaccharide-specific competitive ELISA to motility enrichment culture for detection of *Salmonella* Typhimurium and *S.* Enteritidis in chickens. Of the 3,928 samples collected, 1,085 (28%) were positive with the ELISA

procedure and 1,067 (27%) yielded a *Salmonella* isolate on culture. The sensitivity of the ELISA was 93% and the specificity was 96.7%. The ELISA used in this study would be an acceptable indicator of infection.

The ELISA procedure can be used to detect *Salmonella* from various biological and environmental samples. Benefits of the procedure include speed and low labor requirements compared to culture. Further research on the diagnostic value of the ELISA as a method to detect *Salmonella* in reptiles should be pursued.

Polymerase chain reaction, once considered only a research technique, is being used more frequently in the clinical diagnosis of disease and for epidemiological investigations (Fredricks and Relman, 1999). Polymerase chain reaction assay is an enzyme mediated process used to replicate DNA from an organism with specific oligonucleotide primers that are complimentary to specific nucleotide sequences of the subject organism. Because the PCR technique can be used to create logarithmic copies of microbial DNA from a limited amount of sample, the technique can identify organisms in clinical or environmental samples at levels too low to detect with culture. Several techniques can be used to detect microbial DNA with PCR, including specific PCR, broad-range PCR, multiplexing PCR, nested PCR, and reverse-transcriptase PCR (Fredricks and Relman, 1999). The PCR assay amplifies DNA with a thermostable DNA polymerase in combination with a buffer, magnesium, deoxyribonucleoside triphosphates, and oligonucleotide primers. The primers anneal to complimentary regions on the coding and noncoding strand of DNA. The DNA polymerase attaches to DNA primer complexes and extends the DNA. The copy made in the first cycle serves

as a template for further amplification. Multiple cycles at various temperatures are repeated and the process of disrupting the double-stranded DNA, annealing the primers to the DNA, and extending the DNA, produces a logarithmic increase in the template. There are a number of different techniques used to confirm the presence of target DNA, including gel electrophoresis, DNA sequencing, oligonucleotide probes, and restriction fragment length polymorphism.

The PCR assay is considered to be more sensitive than culture because it can amplify DNA from a single organism or part of an organism under suitable conditions. Specificity of the PCR assay can also be very high depending on the primers used. PCR assay may be prone to false positive reactions if processing of the samples is not performed under sterile conditions. To prevent amplification of contaminant DNA, processing should be conducted in separate pre-and post-PCR rooms. A number of biological inhibitors affect the results of a PCR assay, including blood, blood culture media, urine, sputum, and vitreous humor (Fredricks and Relman, 1999). Sample processing can influence PCR assay results, yielding false negatives if the DNA extraction technique does not release microbial DNA, or if the quantity of DNA available for the reaction is low (Fredricks and Relman, 1999).

All *Salmonella* serotypes are considered potentially pathogenic (Acha and Szyfres, 1987). A diagnostic test that is capable of identifying *Salmonella* to the generic level could be used to screen animals and their environment. Cohen et al. (1993) developed 25 bp oligonucleotide primers to define a 496 bp segment of the histidine transport operon gene of *Salmonella* Typhimurium.

A potential advantage of the PCR assay is supposed to be the ability to detect low numbers of organisms in a sample. Cohen et al. (1994b) calculated the minimal detection numbers of *Salmonella* in experimentally infected feces from horses using the PCR assay described previously (Cohen et al., 1993). The minimal detection limit for PCR was 10^3 to 10^4 CFU/ g feces, compared to the minimal detection limit for culture of 10^2 to 10^3 CFU/ g feces. However, culture results were based on enrichment in selenite broth; in this study, the samples tested with PCR were not enriched. A short enrichment period in selenite may have lowered the minimal detection levels for PCR. PCR diagnosis was more rapid than culture, requiring 10-12 hours for PCR versus two to four days for culture.

Cohen et al. (1994a) compared PCR assay to microbiologic culture for detection of *Salmonella* in poultry litter. Drag swabs collected from 18 poultry houses at nine different broiler farms were tested for *Salmonella* with the PCR assay and culture. Out of 50 samples tested, 47 (94%) of the PCR samples and 29 (58%) of the culture samples were positive for *Salmonella*. The PCR was significantly more sensitive than culture for detecting *Salmonella* in poultry litter.

Cohen et al. (1996) compared a PCR assay to standard microbiological culture for detection of salmonellae from naturally infected equine feces and environmental samples from the teaching hospital at the Texas A&M University, College of Veterinary Medicine. Of the 313 environmental samples collected, eight (3%) were positive based on the PCR assay but none of the cultures detected *Salmonella*. Fecal samples were collected from an outpatient group without enteric signs and a hospitalized group with

gastrointestinal disease. Of the 152 outpatient horses tested, 26 (17%) were positive based on the PCR assay but none of the cultures yielded an isolate. Of the 110 subjects with gastrointestinal disease, 71 (65%) of the samples were positive with the PCR assay, in contrast to only 11 (10%) of the cultured samples. The PCR assay detected all eleven of the cultured isolates. Results indicated that the PCR assay would be a useful technique for epidemiologic investigations in evaluating a population with a low prevalence of *Salmonella*.

2.6 *Salmonella* in Reptiles

2.6.1 Reptile Salmonellosis

Salmonella was first isolated from a lizard (*Heloderma suspectum*) in 1944. (McNeil and Hinshaw, 1946), although an earlier unconfirmed report of an organism consistent with the biochemical attributes of *Salmonella* was isolated from three dead wild-caught Gila monsters in 1939 (Caldwell and Ryerson, 1939). *Salmonella* was first isolated from a gopher snake (*Pituophis catenifer deserticola*) killed on a turkey farm for eating turkey poults. The snake harbored three serotypes: *Salmonella* Panama, *S.* meleagridis, and an unidentified paracolon type (Hinshaw and McNeil, 1944). This was also the first report of multiple serotypes being isolated from the same reptile. Snakes were considered a reservoir for *Salmonella*, and were implicated as the source of the *Salmonella* outbreak in the turkey flock. The first report of a *Salmonella* isolate from a chelonian involved *Salmonella* Newport, which was isolated from the liver, spleen, lungs and intestine of a Galapagos tortoise (*Geochelone giganteas*) at necropsy (McNeil and Hinshaw, 1946).

Clinical signs of *Salmonella* infections in reptiles are variable. In a group of five Gila monsters inoculated intracoelomically with *Salmonella*, four of the animals died within a period of 18 hours and three months (Caldwell and Ryerson, 1939). *Salmonella* was re-isolated from two of the dead Gila monsters, but no gross lesions were observed in any of the lizards. The fifth lizard was unaffected. In a study on horned lizards *(Phrynosoma solare)*, five animals were infected with *Salmonella*, resulting in death of all five animals within twelve days. Splenomegaly was reported in several of the lizards and *Salmonella* was isolated from all five animals at necropsy.

Onderka and Finlayson (1985) sampled 150 reptiles at necropsy for *Salmonella*. Forty-six (51%) of the snakes, twenty-two (48%) of the lizards and one (7%) of the chelonians yielded *Salmonella*. Thirty-one serotypes were isolated from the sampled population. Death in fifteen (17%) snakes and five (11%) lizards was attributed to salmonellosis. Pure cultures of salmonellae have been isolated from snakes with subacute necrotizing enteritis (Onderka and Finlayson, 1985). *Salmonella* has also been identified as the primary pathogen in a boa constrictor (*Constrictor constrictor*) with pneumonia. Reptiles that undergo bacteremia may develop visceral lesions. Hepatitis has been observed in snakes, and nephritis, oophoritis, myocarditis, and aortic valvular endocarditis have been diagnosed in green iguanas with *Salmonella* bacteremia at post-mortem (Onderka and Finlayson, 1985). McNeil and Hinshaw (1946) isolated *Salmonella* Newport and *S.* Sandiego from two Galapagos tortoises at necropsy. *Salmonella* Montevideo was isolated from the ovaries, spleen, and lungs of a Gila monster with exudative pleural pneumonia. *Salmonella* Manhattan was isolated from

the liver, spleen, blood, urinary bladder, and intestine of an iguana (*Iguana iguana*) at necropsy.

Salmonellosis has also been reported in farm-reared crocodilians (Huchzermeyer, 1991; Manolis et al., 1991). Affected animals were lethargic and anorectic. *Salmonella* septicemia was also associated with acute death in farm-raised Nile crocodiles. High stocking density, contaminated diets, and a deficiency in hygiene were considered to be factors predisposing to infection and clinical severity (Huchzermeyer, 1991).

2.6.2 *Salmonella* as a Component of the Indigenous Gastrointestinal Flora

Salmonella are routinely isolated from apparently healthy reptiles. Refai and Rohde (1969) isolated *Salmonella* from 14 of 25 fecal samples from reptiles in the Gizeh Zoological Gardens. Five of the fecal samples contained two serotypes and one fecal sample contained three serotypes. Zwart (1962) examined samples from wild and captive reptiles in Ghana and found that the prevalence of *Salmonella* in lizards (37.5%) was greater than in snakes (29.6%). A collection of reptiles comprising 11 snakes and 3 crocodilians maintained in a natural museum were examined for salmonellae (Kennedy, 1973). Six of the snakes yielded *Salmonella*, whereas the crocodilians were free of infection. Kourany and Telford (1981) collected intestinal samples from 447 wild lizards in Panama, of which 131 (29.4%) were *Salmonella*-positive, with 36 different serotypes identified.

One-hundred and twenty-seven chelonians from the reptile collection at the Bronx Zoo were examined for *Salmonella* by Otis and Behler (1973). Thirty-seven of

the chelonians were *Salmonella*-positive for *S.* Durham. The prevalence in this population is similar to that described for chelonian collections at the Basel, Bern and Zurich zoos (Rudat et al., 1966). The prevalence reported in the chelonian collection at the Frankfurt Zoological Park was much higher (50.5%) than those reported from other institutions (Lie, 1968). Jackson and Jackson (1971) sampled chelonians (N=124) from 9 different zoological parks in the United States and reported an overall prevalence of 12.1%. The differences between these populations may be attributed to methods of sample handling and isolation, and also environmental exposure and contamination.

2.6.3 *Salmonella* in Crocodilians

Crocodilians and green iguanas are the only reptiles routinely raised on farms as a source of meat and leather. Contaminated meat is a potential source of *Salmonella* for humans, and *Salmonella* has been isolated from both captive and wild crocodilians. Scott and Foster (1997) evaluated the prevalence of *Salmonella* in wild and farm-raised American alligators (*Alligator mississipiensis*) from Texas and Louisiana. Two percent (2/71) of the wild alligators were *Salmonella*-positive, compared to 14% (4/29) of the farm-raised alligators. The four positive farm-raised alligators were housed indoors. Generally, indoor confined housing involves a higher stocking density than in outdoor enclosures, and hygiene is frequently inferior.

Eight jejunal swabs (16%) collected from 50 farm-reared Nile crocodiles at necropsy yielded *Salmonella* (Obwolo and Zwart, 1993), with *Salmonella* Arizona identified as the most common serotype (7/8). The source of the *Salmonella* was unknown, but either contaminated food or water was implicated.

The prevalence of *Salmonella* from fecal samples collected from *Crocodylus porosus* and *C. johnstoni* on a farm in the Northern Territories, Australia was 5% (1/20) and 81% (17/21) respectively. The prevalence of *Salmonella* in cloacal samples from these two species collected from a second farm in the same area was 30.5% (46/151) and 20% (21/105) respectively. The differences in recovery rate between the two farm populations may be attributed to contamination of the diet and the environment.

Madsen et al. (1998) collected cloacal and skin cultures from 67 wild Nile crocodiles captured in uninhabited areas. Eighteen (27%)of the crocodiles yielded *Salmonella* on cloacal culture with eight different *Salmonella* serotypes identified. All 67 skin cultures were free of *Salmonella* infection. Antimicrobial susceptibility testing on twelve *Salmonella* isolates demonstrated that three isolates were resistant to streptomycin and nine were of intermediate susceptibility. Antimicrobial resistance is reported in farm-reared crocodilians and is attributed to inappropriate use of antibiotics.

The majority of farm-raised crocodilians are from eggs harvested in the wild and incubated on farms. This is a common practice in the United States for *A. mississipiensis*, in Africa for *C. niloticus*, and in Australia for *C. johnstoni* and *C. porosus*. Feeding farm-reared crocodilians poultry carcasses may result in infection with *Salmonella*. Crocodilians raised for meat may also serve as a source of food-borne *Salmonella* in consumers and appropriate slaughter and processing hygiene should be followed.

2.7 Reptile-Associated Salmonellosis in Humans

2.7.1 Turtle-Associated Salmonellosis

The first case of turtle-associated salmonellosis in humans was reported in 1943 (Boycott et al., 1953) and the frequency of cases increased over the next twenty years (Williams and Heldson, 1965). It was not until 1963 that the first case of turtle-associated salmonellosis in a child was reported (Hersey and Mason, 1963). *Salmonella* Hartford was recovered from a 7-month old infant with diarrhea, vomiting and fever. An investigation of the infant's environment resulted in the isolation of the same serotype from the family's pet turtle. The increased frequency of turtle-associated salmonellosis in children was of concern to both state and federal health officials.

On January 1, 1968 the Washington State Board of Health issued a regulation restricting the sale of turtles that were not certified *Salmonella*-free and effectively halted the pet turtle retail trade in the state (CDC, 1971). The number of turtle-associated salmonellosis cases decreased in the Seattle, WA area after the policy was instituted. Salmonellosis attack rates for children under ten years of age in the State of Washington were lower after 1968 as compared to 1966 and 1967, while the attack rate for children in this same age group in the United States increased (Lamm et al., 1972).

In an attempt to estimate the magnitude of turtle-associated salmonellosis in the United States, retrospective surveys of laboratory confirmed cases were reviewed to determine frequency of turtle ownership with data from various state and county health agencies, including Utah; Atlanta, Georgia; Santa Clara County, California; and Seattle, Washington (Lamm et al., 1972). A retrospective case-control study was also conducted

on cases reported in the State of Connecticut. Twenty-four percent of the salmonellosis cases in Connecticut were associated with an exposure to a turtle, compared to 2% of the controls. The findings in the other parts of the country varied, with salmonellosis cases in Santa Clara, California reporting an association with turtles in 18% of the cases, 15.6% in Utah, 11.6% in Seattle, and 10.9% in Atlanta (Lamm et al., 1972). This information was than combined with previous turtle-associated salmonellosis reports in an uncontrolled study in Minnesota (turtle exposure: 25%; Williams and Heldson, 1965) and a controlled study in New Jersey (turtle exposure cases: 22.6%; controls: 5.7%; Altman et al., 1972). An estimate of the number of turtle-associated salmonellosis cases in the United States was then developed. The proportion of juvenile salmonellosis cases associated with exposure to turtles was averaged among the seven sites to yield a mean of 18.2%. This average was then applied to the estimated number of salmonellosis cases in the United States (2,000,000; Aserkoff, 1970), assuming that turtles were present in 4.2% of U.S. households. The estimate of the total number of households at risk was determined by a calculation derived from the number of turtles sold in a given year (1971: 15,000,000) and the number of households in the U.S. (1971: 60,000,000). The estimate of 4.2% corresponded to the number of households that maintained a turtle in the Connecticut and New Jersey studies (Lamm et al., 1972). Based on these reference parameters, approximately 14% (280,000) of the salmonellosis cases in the U.S. in 1971 were turtle-associated.

The Federal government realized the significant health risk associated with pet turtle ownership and enacted regulatory measures similar to those enforced in

Washington. In 1972, the Food and Drug Administration required certification verifying *Salmonella*-free status for the interstate transport of pet turtles. This program was found to be ineffective. A study conducted by the Center for Disease Control concluded that 38% of the animals certified to be *Salmonella*-free were contaminated (CDC, 1974). In 1975, the Food and Drug Administration banned interstate shipment of all turtle eggs and live turtles with a carapace length less than 10.2 cm. The decision to restrict the sale of turtles with a carapace length less than 10.2 cm was based on the assumption that these animals would be less desirable to young children. Enforcement of this policy resulted in a 77% reduction in the incidence of cases in those states without indigenous production of turtles (Cohen et al., 1980). The number of cases in Louisiana and Mississippi, two prominent turtle producing states, remained unchanged during 1970-1976, in contrast to a significant decrease in other non-turtle producing states (Cohen et al., 1980).

The federal ban did not restrict exportation of turtles from the U.S., and *Salmonella* Pomona was isolated from turtles shipped from Louisiana to Guam and Yugoslavia (Tauxe et al., 1985), Japan (Fujita et al., 1981), Great Britain (Borland, 1975), France (Sanchez et al., 1988), and Israel (Chassis, 1986). In 1984, an illegal shipment of turtles to Puerto Rico was responsible for an outbreak of *Salmonella* Pomona. Health officials estimated that 15% of all infant salmonellosis was attributed to the illegal shipment (Rigau-Perez, 1984).

Exceptions to this 1975 law were made for marine turtles and educational and scientific institutions. Violators of this law are provided a written demand to destroy the

animals under FDA supervision within 10 days. Violators are also subject to a fine not more than $1000 and/or imprisonment of not more than one year for each violation. Herpetoculturists that breed exotic chelonians have avoided prosecution by selling animals under the guise of educational animals. Currently, there are several hundred reptile swap meets a year in the U.S. where chelonians with a carapace length less than 10.2 cm can be purchased.

Attempts to reduce or eliminate *Salmonella* in turtles with antimicrobials was initiated after the FDA ban was implemented in 1975. Treatment of hatchlings with oxytetracycline in their tank water for up to 14 days alleviated shedding in treated turtles, but did not affect systemic infection (Siebling et al., 1975). Treatment of the freshly laid eggs with oxytetracycline or chloramphenicol with a temperature differential egg dip method was successful at eliminating *Salmonella* in eggs less than one day old, but did not clear eggs greater than two days old (Siebling et al., 1975). Large-scale experimentation on commercial turtle farms with surface decontamination and pressure or temperature differential treatment of eggs with gentamicin dip solutions for eggs greater than 2 days old, followed by hatching the eggs on *Salmonella*-free bedding, substantially reduced *Salmonella* infections and shedding rates in hatchling turtles (Siebling et al., 1984). Forty percent of the eggs not treated with the gentamicin were found to harbor *Salmonella,* whereas only 0.15% of the treated eggs were positive. Legislative implementation of this concurrent method of surface decontamination and gentamicin treatment by the Louisiana Department of Agriculture in 1985 was hailed as victory by turtle farmers. Unfortunately, use of gentamicin and the other antimicrobials

has led to an even greater concern due to the development and persistence of antimicrobial resistant strains of *Salmonella*.

The occurrence of *Salmonella* in red-eared turtle (*Pseudemys scripta elegans*) eggs imported to Canada from four different Louisiana turtle farms in 1988 was examined, and of the 28 lots tested, six (21%) lots from three of four exporters were *Salmonella* positive (D'Aoust et al., 1990). Of the 37 *Salmonella* strains isolated, 30 (81%) were gentamicin resistant (D'Aoust et al., 1990). Similar results have been reported from samples collected directly from the farms in Louisiana. Shane et al. (1990) collected environmental samples and live hatchlings directly from two Louisiana turtle farms. Isolates of *S.* Arizonae and *S.* Poona collected from turtles at one of the farms were resistant to erythromycin, gentamicin, tetracycline and triple sulfa. Pond water samples from both farms showed similar antimicrobial resistant patterns to erythromycin. In 1988, 115 batches of turtle hatchlings were submitted from 28 farms to the Louisiana Department of Agriculture and Forestry for analysis (Shane et al., 1990). Five (4.3%) *Salmonella* isolates were obtained. Four of the organisms were submitted for serotyping; three were *S.* Arizona and one as *S.* Poona. All four isolates were resistant to erythromycin, gentamicin, tetracycline, and triple sulfa. These findings suggest that these animals may pose a significant human health risk and further marketing of these animals should be curtailed.

Reports of certified *Salmonella*-negative hatchling turtles testing *Salmonella*-positive when arriving in the importing country have been on the rise since the advent of egg sanitation and antimicrobial dip treatments (D'Aoust et al., 1990). A reason for this

discrepancy has been attributed to the sample size required for testing (Shane et al., 1990). In 1972, United States Federal regulations established to screen hatchling turtles for *Salmonella* required that 60 turtles be submitted for culture to a certified diagnostic laboratory. At the time of the regulations the prevalence of *Salmonella* in turtles was approximately 40% (Cohen et al., 1980). However, the advent of egg sanitation and antimicrobial treatment methods reduced the apparent prevalence below 0.2% (Siebling et al., 1984). A standard normal approximation of a binomial distribution used to estimate sample size has shown that there is only a 26% probability of detecting *Salmonella* in a population of turtles with a prevalence of 0.5% (Shane et al., 1990). These findings suggest that a larger sample size of turtles should be submitted to ensure that false-negative batches are not exported.

2.7.2 Reptile-Associated Salmonellosis: 1990-Present

The incidence of reptile-associated salmonellosis cases in humans has increased dramatically during the past decade (CDC, 1996). In 1996, the Centers for Disease Control estimated that reptiles accounted for 3-5% of the 2-6 million cases of human salmonellosis in the U.S. (Cambre and McGuill, 2000). In most documented reptile-associated cases of salmonellosis, the strain of *Salmonella* isolated from the patient was common to the pet reptile suggesting the source of infection (Meehan, 1996). The increased incidence of reptile-associated salmonellosis has been associated with the increased popularity of these animals as pets during the past decade. From 1989 to 1993 imports increased 82%, from 1.1 million to 2.1 million animals (United States Fish and

Wildlife Service, 1993). Green iguanas accounted for the largest proportion with imports increasing by 431% from 143,000 to 760,000.

2.7.3 Reptile-Associated Salmonellosis: Case Reports

In 1990, two isolates of *Salmonella* Marinum were reported by the Indiana State Board of Health, Disease Control Laboratory, from two infants residing in different counties, 80 miles apart (CDC, 1992a). The parents of both children owned a pet iguana. The iguanas were purchased from different pet stores, but were derived from a common distributor and importer. In both cases, the infants responded to supportive care and appropriate antimicrobial therapy. In one of the cases, *S.* Marinum was isolated directly from the iguana and in the other case from the iguana's enclosure. The infants were reported not to have direct contact with the iguana, suggesting that infection may occur in the absence of direct contact with an animal excreting *Salmonella.*

In 1992, a rare strain of *S.* Poona was isolated from an 8-week old infant presented to a pediatric clinic with bloody diarrhea, flatulence, and fever (CDC, 1992b). The infant was provided supportive care and appropriate antimicrobial therapy and the symptoms resolved. Although the family owned a pet python, samples collected from both the animal and its enclosure were negative. The family had owned a Savannah monitor (*Varanus exanthematicus*), which had been traded for the snake one month previous to the diagnosis of salmonellosis. The cage substrate and water bowl used by the monitor over a month earlier were positive for *S.* Poona. The father was the only individual to have contact with the animal and was reported to have cleaned the large

enclosure. The cage accessories, including the hot rock and water bowl, were cleaned in the kitchen sink. This case reinforces the persistence of *Salmonella* in the environment and the need for thorough disinfection.

In October 1995, a 3-week old infant died in Indiana from *S.* Poona. The same serotype was isolated from a pet iguana. The parents claimed that the infant did not have direct contact with the pet. This was the first reported case of a human fatality attributed to a *Salmonella* isolate from a pet iguana (CDC, 1996).

Since these reports were first published, additional cases involving infants, children, immunocompromised individuals, and healthy adults have been described (CDC, 1996). During 1994-1995, health departments in 13 U.S. states reported over 60 cases of individuals with unusual serotypes of *Salmonella* in which patients had either direct or indirect contact with reptiles. The increased number of reports created a special concern for public health officials and prompted further study into the association of "reptile" specific *Salmonella* serotypes with cases of human salmonellosis.

2.7.4 Epidemiology of Reptile-Associated Salmonellosis in Humans

Atypical serotypes of *Salmonella* have been anecdotally associated with reptile ownership. Cieslak et al. (1994) defined and described the epidemiology of reptile-associated serotypes (RAS) in the United States. Reptile-associated serotypes of *Salmonella* were defined as those in which reptilian sources composed the majority of the reports of non-human isolates reported to the CDC between 1981 and 1990. Human isolates of RAS reported to the CDC between 1970 and 1992 were analyzed and incidence rates calculated by age, sex, state and year. A 1991 American Veterinary

Medical Association Survey was used to compare reptile ownership to rates of RAS isolation by state. The annual incidence of RAS increased from $2.4/10^7$ persons per year in 1970 to $8.4/10^7$ persons per year in 1992. This rise in cases represents a total of 150 new cases per year based on United State population estimates. Pet reptile ownership has been reported to be increasing (7.3 million reptiles; CDC, 1995), however estimates of turtle sales in 1971 (15,000,000 turtles; Lamm et al., 1972) far exceed these more recent estimates. The increased incidence of RAS may be associated with improved clinical diagnostic techniques, a higher carriage rate in squamates (non-chelonian reptiles), or the duration of exposure to the pet. The average life span of an aquatic turtle in captivity in 1971 was estimated to be less than two-months when these pets were marketed before the 1975 FDA ban on interstate transport (Lamm et al., 1972). The average longevity of squamates in captivity in 2000 is likely greater than 2 months because of improved husbandry techniques. The number of male salmonellosis cases ($4.1/10^7$ /year) was higher than females ($3.5/10^7$ /year). Although no study has been performed to evaluate gender differences associated with pet reptile ownership, anecdotal reports suggest boys would be more likely to own these animals as pets. The seventeen states with the highest incidence of RAS isolates from humans also had the highest rate of reptile ownership. The incidence of infection was higher in infants ($66.1/10^7$ /year) than in individuals > 1 year of age. Infants also accounted for a greater number of RAS isolates (27.2%) than other *Salmonella* serotypes (18.8%). The higher incidence of infection in infants is contrary to the findings with turtle-associated salmonellosis cases during the early 1970's. In the turtle-associated cases, case-patients

were between 1 to 9 years of age and had direct contact with the reptile, whereas in the more recent cases (1990's), infants are reported to have no direct contact with the pet reptile.

In 1993, three green iguana-associated salmonellosis cases were reported to the New York State Department of Health (NYSDH) during a 2-month period (Ackman et al., 1995). The cases were notable because they involved rare *Salmonella* Matadi and *S.* Poona. The *S.* Matadi was isolated from the stools of two teenage boys that had handled a green iguana. The *S.* Poona was isolated from the cerebrospinal fluid of a 6-week old infant. These cases prompted health officials to question whether specific *Salmonella* serotypes are associated with exposure to reptiles.

A matched case-control study was performed with the 1993 New York State *Salmonella* surveillance data base (Ackman et al., 1995). Case selection included those individuals with *Salmonella* serotypes common to reptiles or characterized in reports of reptile-associated salmonellosis. Reptile-associated serotypes of *Salmonella* were defined as those which reptilian sources composed the majority of the reports of non-human isolates reported to the CDC between 1981 and 1990 (Cieslak et al., 1994). The RAS included 35 serotypes of *Salmonella* type I; all serotypes in II, III, and IV; and ten other serotypes linked to human RAS cases. Each case was matched by age (< 5 years of age within 2 years; 5-21 years of age within 3 years; and > 21 years of age within 10 years) and date of diagnosis (within 30 days) to one or two controls, comprising cases of *Shigella* infection reported to the NYSDH in the same year. Telephone surveys were conducted on all available cases and controls to acquire data on symptoms,

hospitalization, pet ownership, exposure to reptiles, and dietary habits. Of the 1,362 *Salmonella* serotypes, 42 (3%) were considered RAS, of which 24 (57%) interviews were conducted. Twelve (50%) of the cases reported reptile ownership, as compared to only 2/28 controls (matched odds ratio 6.6; 95% confidence interval: 1.4-31.0). Ten of the cases had specific contact with iguanas, whereas none of the controls reported having contact with an iguana. It has been estimated that approximately 5% of all salmonellosis cases are reported (Chalker and Blaser, 1988). Using this estimate, there would have been in excess of 700 RAS cases in New York State during 1993. The findings suggest that reptile-associated salmonellosis is more than an incidental occurrence.

Outbreaks of RAS are rare, despite the fact that 95% of the 174 zoos and aquariums in North America affiliated with the American Zoo and Aquarium Association exhibit reptiles v(Miller, 1997). In January 1996, *Salmonella* Enteritidis was isolated from the feces of several children living in Jefferson County, Colorado (Friedman et al., 1997). The only common link among these patients was that they had visited an exhibit of Komodo dragons held over a 9-day period at the Denver Zoological Gardens. An epidemiologic investigation (matched case-control study) was conducted to assess the extent of the outbreak. A confirmed case was defined as an individual that had gastrointestinal disease and an isolate of *S.* Enteritidis from a fecal sample during the specified time period (January 11-21, 1996). A suspect case was one that attended the exhibit during the specified time period and experienced gastrointestinal disease. A secondary case was defined as an individual classified as a case or suspect, but who

became ill subsequent to a case-patient in the household. Controls were selected from the list of zoo patrons who participated in a promotional event that took place during the exhibit. Cases and controls were matched according to one of 9 age groups and the day they attended the exhibit. A telephone interview was performed to identify cases and controls and to elicit demographic data, medical history, exposure, and activity at the zoo. Culture samples were also collected from the Komodo dragons, their environment, food source (rats), and the zoo keepers working with these animals.

There were 39 culture-confirmed cases and 26 suspect cases. Forty-eight case individuals, comprising 33 culture-confirmed and 15 suspects, were the first to become ill in their households. The median age of the patients was 7 years (range: 3 months-48 years); 53 (82%) were under 13 years old and 34 (55%) were male. The median time until the onset of disease was 3.5 days. *Salmonella* Enteritidis was isolated from only one of the four Komodo dragons at the exhibit. The phage type 8 isolate was common to the case patients. The same organism was also isolated on three occasions from the barrier wall that separated the animals and the visitors. Visitors were allowed to rest their hands and elbows on the barrier surface, which was also accessible to the animals. Direct contact with the reptiles was limited, with only two controls touching an animal. Twenty-six cases were matched to 49 controls for the case-control study. There was a significant risk associated with touching the barrier that housed the dragons (Odds Ratio: 4.0; 95% Confidence Interval: 1.2-13.9). Hand washing after visiting the exhibit or prior to the next meal was found to be protective (OR: 0.1; 95% CI: 0.02-0.5). There was no difference between the groups when comparing the risk of eating food purchased

at zoo concessions or touching a reptile skin on display at the exhibit. This report described the first known outbreak of reptile-associated salmonellosis at a zoologic park and reinforces the concern that transmission of *Salmonella* from reptiles to humans may occur thorough environmental contamination.

2.8 Methods to Eradicate or Suppress *Salmonella*

2.8.1 Enrofloxacin: Structure, Activity, Clinical Use, and Adverse Effects

Enrofloxacin is a member of the family of 6-fluoro-7-piperazinyl-4-quinolones (Hooper and Wolfson, 1985). Enrofloxacin is highly lipophilic and the addition of a carboxic acid and a tertiary amine contribute to the amphoteric properties of enrofloxacin (Vancutsem et al., 1990). The fluoroquinolones are bactericidal compounds with activity against both Gram-positive and Gram-negative pathogens which may be intracellular pathogens (Hooper and Wolfson, 1985; Scheer, 1987). Modification of the 4-quinolone ring has enhanced the antimicrobial activity. Oral bioavailability of enrofloxacin is excellent in monogastric mammals and preruminant calves, with up to 80% of the ingested dose being absorbed into systemic circulation (Vancutsem et al., 1990). Oral absorption of fluoroquinolones is rapid, with peak serum concentrations achieved one to two hours after administration (Parpia et al., 1989). Fluoroquinolones do not readily complex with plasma proteins, which enables metabolites to cross cell membranes readily. In humans, approximately 10-40% of the fluoroquinolones are bound to plasma proteins (Vancutsem et al., 1990). The fluoroquinolones are widely distributed throughout the body, including the kidneys, liver, bile, prostate, uterus and fallopian tubes, bone, and inflammatory tissues (Montay

et al., 1984). Excretion of the fluoroquinolones is primarily through the kidneys with secondary excretion through the liver (Montay et al., 1984; Vancutsem et al., 1990).

Fluoroquinolones alter the action of bacterial DNA gyrase, a type II topoisomerase (Vancutsem et al., 1990). This enzyme is involved in unwinding, cutting and resealing DNA. There are two subunits to DNA gyrase: Subunit A and subunit B and fluoroquinolones act on the nalA locus of the subunit A. Inhibition of the gyrase leads to rapid cell death in bacteria. The concentration of fluoroquinolones required to alter the DNA of mammalian cells is two orders of magnitude higher than the concentration against bacterial DNA (Oomori et al., 1988; Hussy et al., 1986).

The metabolism of enrofloxacin varies among species. Although enrofloxacin is an active antibiotic, biotransformation to ciprofloxacin may also occur. Biotransformation of enrofloxacin includes N-dealkylation, glucuronide conjugation to the nitrogen in the para position of the piperazinyl ring, oxidation in the ortho position to substituted amine, and opening of the piperazinyl ring (Vancutsem et al., 1990).

The elimination half-life of enrofloxacin varies among species. Chickens have a prolonged half-life (7.3 hours) in comparison to mammals, including canines (2.1 hours), calves (1.2 hours), and horses (3.3 hours) (Vancutsem et al., 1990). The elimination half-life of enrofloxacin is much longer in ectotherms, such as reptiles.

Enrofloxacin has a biphasic concentration-response curve. (Diver and Wise, 1986). In the first phase, the proportion of bacteria killed increases as the concentration of enrofloxacin is increased. In the second phase, bacteria are killed at a lower rate as the concentration of enrofloxacin is increased.

Selection for resistance to the fluoroquinolones occurs from chromosomal mutations, creating gyrase modifications, or alterations in permeability (Easmon and Crane, 1983; Piddock and Wise, 1989). No plasmid resistance has been demonstrated. Mutants develop resistance to other fluoroquinolones and other antimicrobials, including cephalosporins, chloramphenicol, and tetracyclines (Neu, 1988; Bellido and Pechere, 1989).

The adverse effects associated with fluoroquinolones are primarily associated with immature cartilage, the urinary and gastrointestinal tracts, and the central nervous system. Arthropathies have been reported in immature rats, beagles, guinea pigs, and foals (Kato and Onodera, 1988; Burkhardt et al., 1990; Bendele et al., 1990; Berg, 1988). The cartilaginous surfaces of the femur, the humerus, and the tibial tarsal bone are the primary sites where quinolone-induced arthropathies occurred in beagle pups (Burkhardt et al., 1990). The most common histologic findings in quinolone-induced arthropathies are erosions of the articular cartilage (Burkhardt et al., 1990; Kato and Onodera, 1988; Bendele et al., 1990). Histologic lesions may be detected two days after treatment. Fluoroquinolones can achieve a high concentration in the urine, as the kidneys are the primary route of excretion for fluoroquinolones. Because the fluoroquinolones have low solubility in water, they crystallize in acidic urine (Vancutsem et al., 1990). Crystalluria could be a problem in carnivorous animals fed a high-protein diet. The adverse gastrointestinal effects associated with the fluoroquinolones include nausea, vomiting, and abdominal cramping (Schluter, 1987). The descriptions of adverse effects associated with the central nervous system have been

documented in human patients (Ball, 1986). Changes in behavior, including psychosis, headaches, hallucinations and seizures have been reported after treatment with 250 to 500 mg ciprofloxacin. There have been no adverse effects from enrofloxacin treatment reported in reptiles.

Fluoroquinolones are highly effective bacteriocides with relatively low minimum inhibitory concentrations (MIC). Clinical *Salmonella* Arizonae isolates evaluated at the University of California, showed MIC values in 90% of the isolates ranging between 0.128 μg/ml to 1.0 μg/ml (Vancutsem et al., 1990). Berg (1998) reported that MICs demonstrated by 96% of *Salmonella* isolates from clinical samples were less than 0.125 μg/ml. *Salmonella* isolates from urinary tract infections also yielded low MIC levels for ciprofloxacin (0.06 μg/ml; Vancutsem et al., 1990). The systemic distributions of enrofloxacin and ciprofloxacin, in combination with the low MIC levels required to eradicate *Salmonella* and the levels of enrofloxacin achieved in the serum and tissues, suggest that these compounds would be beneficial for treating a prospective patient with a *Salmonella* infection.

Enrofloxacin has been evaluated as a method to eliminate *Salmonella* infections in cattle and poultry. Enrofloxacin administered to cattle at 5 mg/kg/day for ten days eliminated *Salmonella* in more than half the subjects (Spieker, 1986). The *Salmonella* isolates from the cattle that remained infected after treatment were still sensitive to enrofloxacin. Calves experimentally infected with *Salmonella* Typhimurium were cleared of the infection after being treated with enrofloxacin at a dose of 5 mg/kg/day for six days (Bauditz, 1987). Enrofloxacin administered to poultry in drinking water at

50 ppm for five to ten days was effective against experimental *Salmonella* Typhimurium infection in broilers and turkeys (Bauditz, 1987). Broilers reinfected with *Salmonella* Typhimurium fourteen days after discontinuing treatment of 100 to 200 ppm enrofloxacin were reinfected at a similar rate (Bauditz, 1987). Enrofloxacin, when administered under controlled conditions, was effective at eliminating *Salmonella* from domestic livestock and poultry.

Enrofloxacin has been used to treat bacterial infection in reptiles because it is active against most of the Gram-positive and Gram-negative bacteria affecting these species. Pharmacokinetic studies to detect the rates of absorption, metabolism, distribution, and excretion of enrofloxacin in reptiles are limited. Enrofloxacin administered either intramuscularly (IM) or *per os* (PO) at a dose of 10 mg/kg IM or PO at 5 mg/kg in Savannah monitors (*Varanus exanthematicus*) resulted in minimal conversion to ciprofloxacin (Hungerford et al., 1997). The terminal elimination half-life was 40 hours for the IM and PO routes at 5 mg/kg and 36 and 24 hours for IM and PO routes at 10 mg/kg. The peak plasma concentration for the IM and oral routes at 10 mg/kg were 10.5 μg/ml and 3.6 μg/ml respectively. The peak serum concentrations recorded in the monitor lizard would be adequate to treat bacteria with high MIC values. Enrofloxacin administered IM at 5 mg/kg to Burmese pythons (*Python molurus bivittatus*), resulted in a significant conversion to ciprofloxacin (Young et al., 1997). The peak serum concentration of enrofloxacin in the python was 1.66 μg/ml. The mean terminal half-life was 6.37 hours. The 5 mg/kg dose would be effective against bacteria

with MIC values of 0.2 μg/ml. There have been no studies evaluating the pharmacokinetics of enrofloxacin in iguanas.

2.8.2 *Salmonella* Eradication Using Competitive Exclusion Microbes

Competitive microbial exclusion is a phenomena allowing specific microbial constituents of the intestinal flora to inhibit the multiplication of other bacteria, including pathogens, and to inhibit colonization by competing for receptor sites on enterocytes (Greenberg, 1969). The concept of competitive exclusion (CE) was documented by Metchnikoff (1908) who proposed that beneficial organisms, such as *Lactobacillus*, could displace pathogens, improve intestinal health and prolong life. Since this time, studies on CE have been evaluated with single and mixed populations, and defined and undefined cultures, to control enteropathogens in humans and animals.

Food-borne illness in humans attributed to consumption of *Salmonella*-contaminated poultry has increased since the 1970's, prompting studies to evaluate techniques to reduce salmonellae colonization in poultry (O'Brien, 1990). The CE technique has been researched extensively in the poultry industry as a method to prevent colonization of the intestinal tract of chicks and poults with salmonellae. Nurmi and Rantala (1973) and Rantala and Nurmi (1973) applied this technique to poultry to control an outbreak of *Salmonella* Infantis in broiler flocks responsible for food borne outbreaks in Finland. It was determined that chicks are most susceptible to *Salmonella* colonization during the first week post-hatch, before establishment of a stable indigenous microflora. In these naive birds, as little as one to ten *Salmonella* organisms in the crop led to colonization. Attempts to exclude *Salmonella* with *Lactobacillus* were

unsuccessful. An undefined *Salmonella*-free mixed culture derived from adult birds was administered orally to the chicks and provided protection against *Salmonella* Infantis.

The mechanisms used by bacteria to exclude or reduce the growth of a enteropathogen are poorly understood. Rolfe (1991) suggested four possible mechanisms, including: 1) creation of a microecology that is hostile to other bacterial species, 2) elimination of available bacterial receptor sites, 3) synthesis of antibacterial compounds, and 4) depletion of essential nutrients. An acidic pH in the intestinal tract can reduce survival of *Salmonella* and other Enterobacteriaceae (Meynell, 1963), and microbes synthesizing volatile fatty acids can reduce intestinal pH levels and inhibit colonization by enteropathogens. Microbes that block receptor sites on enterocytes can effectively reduce the rate of adhesion of pathogens and the invasion of host cells. Antimicrobial substances, such as reuterin, are natural compounds produced by indigenous microbes that can destroy pathogens. Microbial competition for available nutrients may inhibit the establishment and subsequent multiplication of a pathogen, although many organisms can utilize alternative metabolic pathways to avoid these limiting factors.

Defined CE cultures consist of organisms classified by the U.S. Food and Drug Administration in the category of "generally regarded as safe" (GRAS). Currently, there are 42 GRAS organisms, principally species of *Lactobacillus*, that may be used in defined CE cultures (Corrier and Nesbit, 1999). The mechanism of action used by defined organisms to reduce or control *Salmonella* is poorly understood. Most defined cultures consisting of a single or limited number (<14) of organisms afford limited or

negligible protection (Stavric et al., 1992). A recent study with *Lactobacillus reuteri* reduced colonization of *Salmonella* and *E. coli* in chicks and turkey poults (Edens et al., 1997). *Lactobacillus reuteri* produces reuterin, a broad spectrum antimicrobial, and has been shown to inhibit at least 25 genera of microbial enteropathogens found in mammalian and avian intestines (Chung et al., 1989). Reuterin concentrations as low as 10-30 ug/ml can destroy *Salmonella*, *E. coli*, and *Campylobacter* within 30-40 minutes (Edens et al., 1997). Defined cultures consisting of large numbers of organisms (>14) representing different genera have been shown to provide protection against experimental *Salmonella* colonization (Hudault et al., 1985; Impey et al., 1982; Stavric et al., 1985).

Undefined cultures are routinely prepared from the intestinal microflora of apparently healthy chickens. Continuous-flow culture systems have been used to maintain indigenous chicken flora in a steady-state culture system (Nisbet et al., 1994). A characterized continuous flow culture (CF3) of cecal anaerobes consisting of twenty-nine bacterial isolates comprising fifteen facultative anaerobes and fourteen obligate anaerobes representing ten different genera was patented (Nisbet et al., 1995) and licensed for commercial manufacture (BioScience Division, Milk Specialties, Dundee, IL). This product is effective against experimental *Salmonella* Typhimurium colonization (Corrier et al., 1995a) and challenge with environmental *Salmonella* in commercially reared broilers (Corrier et al., 1995b).

The use of undefined competitive exclusion cultures has raised concerns regarding the potential transmission of animal or human pathogens. Although undefined

cultures contain unidentified organisms, they can be demonstrated to be free of specific animal and human pathogens (Blankenship et al., 1993). The safety of these products has been confirmed in both laboratory and field trials (Stavric and D'Aoust, 1993).

Field application of CE products have provided acceptable results. A commercial undefined CE product (Broilact; Orion, Turku, Finland) which is routinely used to treat broiler chicks in Finland reduced colonization of *S.* Enteritidis PT4 and prevented invasion of viscera (Schneitz, 1992). Newly hatched broiler chicks were sprayed with the commercial product and then exposed on the following day to experimentally infected (10^3 *S.* Enteritidis) chicks serving as "seeders". Chicks were sacrificed twelve days after challenge and the average population of salmonellae in the caeca of the infected seeder chicks was determined to be 6.7 log $units_{10}$ (U)/g compared to 3.3 log $units_{10}$ (U)/g in the group treated with the CE culture. Pooled samples of gall bladder, liver, heart and spleen, were *Salmonella*- positive in the infected group but negative in the CE treatment. Similar results were observed in a study conducted in England (Mead, 1991). Newly hatched chicks treated with an undefined CE culture were exposed to experimentally infected (10^4 *S.* Enteritidis) chicks. A reduction in the number of salmonellae isolated from the caeca was reported in the CE treatment when compared to an experimentally infected group. Chicks treated with a commercial CE culture (Aviguard; Bayer, Mexico City, Mexico) in Mexico yielded similar results (Cameron et al., 1996).

The administration of fructooligosaccharides to control *Salmonella* have been studied extensively, with generally inconclusive results (Stavric and D'Aoust, 1993).

The majority of salmonellae do not utilize lactose, in contrast to indigenous intestinal microbes which metabolize lactose as a primary carbon source. In theory, the addition of dietary lactose should provide a nutrient source for the indigenous flora to compete with salmonellae. The metabolism of lactose produces an acid, which can reduce the intestinal pH and further affect the survival of salmonellae. The addition of mannose or lactose to drinking water of chicks for 10 days reduced colonization of salmonellae, although dextrose, maltose, and sucrose exerted no effect (Oyofo et al., 1989). The administration of lactose to market age broilers was associated with an increased prevalence of *Salmonella* (Waldroup et al., 1992). More consistent results have been reported when a fructooligosaccharide is combined with a CE culture. The addition of lactose at 5% to the diet (wt/wt) of Leghorn chicks in combination with an undefined CE culture, enhanced resistance to colonization with *S.* Enteritidis to a greater extent than CE culture or dietary lactose alone (Corrier and Nisbet, 1991). A subsequent study further supported these findings (Corrier et al., 1994). A defined CE culture consisting of 11 indigenous strains of cecal bacteria was maintained in a continuous flow culture with lactose as a primary carbon source. The culture was administered by crop gavage to Leghorn chicks on the day of hatch. Controls received either lactose or the defined CE culture without lactose. Chicks were challenged with *S.* Enteritidis PT13 on the following day. When either dietary lactose or the defined CE culture was administered separately, a reduction in salmonellae colonization was determined but the treatments were not consistently protective. The combination of the two products was effective in

preventing colonization. The cost of lactose supplementation and the diarrhea which occurs at the levels required to inhibit colonization prevented commercial application.

The technique used to administer a CE culture influences efficacy. Corrier et al. (1994) evaluated four routes of delivery, including drinking water, encapsulation in alginate beads, whole body spraying, and crop lavage. The administration of the CE culture into the crop ensured direct delivery, but was considered impractical for commercial units. All four treatment groups were challenged with *S.* Enteritidis PT 13 two days after treatment and the cecal contents were sampled seven days later. A reduction in cecal colonization was observed as a result of crop lavage, administration in drinking water, and whole body spraying (0-1.1 $\log_{10}$ U/g) when compared to controls (4.91-5.35 $\log_{10}$ U/g). No difference was detected between the group receiving CE culture in alginate encapsulated beads and the controls. The application of CE culture through whole body spraying (Corrier et al., 1995a) or in drinking water (Mead, 1991), or a combination of the two methods (Blankenship et al., 1993), has been successful in reducing salmonellae colonization in large scale field trials.

Studies to eliminate salmonellae from the intestinal tract of mature chickens have been attempted using a combination of an antibiotic followed by a CE culture. Fowler and Mead (1990) used this sequential combination therapy to prevent re-infection in 250,000 adult (nineteen week old) breeder chickens, in addition to turkeys and ducks in 22 trials over a two year period (1986-1988). Birds received various antibiotic supplements in feed for 14 days. Chickens were treated with chlortetracycline (200 ppm) or furazolidine (400 ppm), turkeys received neomycin (200ppm) and

chlortetracycline (200ppm), and ducks were supplemented with neomycin (200 ppm). The day after antibiotics were discontinued, the birds received a CE culture in their drinking water. In 20 of the 22 trials, subjects were protected from reinfection and the flocks remained *Salmonella*-negative over a subsequent 3-month period following treatment. In the Netherlands, 32 breeder flocks with confirmed *S.* Enteritidis infection were enrolled in a study. The birds received antibiotics followed by a commercial undefined CE culture (Aviguard; Bayer, Manheim, Germany). Bacterial cultures were collected from the breeders and their progeny. Seventy-two percent of the treated birds were *Salmonella*-negative after one treatment (antibiotic-CE culture) and 93% of the birds were negative after two treatments.

Research to elucidate the mechanism(s) of CE cultures in providing protection and identifying "determinant factors" in inhibition requires further study (Stavric et al., 1985). For CE-based control to be effective it must be integrated into a comprehensive program of administration and management.

The CE culture may be a valuable method to reduce or prevent salmonellae infection in green iguanas. Application of a CE culture in the form of a water spray or direct gastric lavage are practical methods of administration as animals can easily be captured and treated. The combined antibiotic-CE culture method may useful in eliminating salmonellae from adult breeder animals or hatchling animals subsequent to importation or purchase. Further studies are required to evaluate the efficacy of this control method in iguanas.

2.8.3 *Salmonella* Eradication Using Vaccination

Salmonella colonization of the intestinal tract in domestic cattle, poultry, and swine is widespread. Once *Salmonella* becomes endemic on a farm it can be readily transmitted by vermin and insects, and by suboptimal biosecurity and management. Application of antimicrobials, competitive exclusion cultures, and procedures that promote hygiene to control *Salmonella* in domestic species have achieved mixed results. Vaccination programs to protect breeding animals and their progeny has been introduced in the poultry industry to suppress and ultimately eliminate *Salmonella*.

An understanding of the pathogenesis of *Salmonella* is necessary to predict potential efficacy of a vaccine. *Salmonella* ingested by the host colonize the intestine and multiply within the lumen or attach and invade enterocytes. The primary route of entry into the systemic circulation is through the mucosa and epithelial lining of the intestine (Groisman et al., 1990). Host cells respond to invasion of the epithelial cells by producing phagolysosomes and mobilizing macrophages to the area. In response, salmonellae produce heat-labile enterotoxins that increase cyclic AMP, and cytotoxins that inhibit protein synthesis by the host cell (Groisman et al., 1990). Once the salmonellae enter systemic circulation, they localize and proliferate in the reticuloendothelial system. Salmonellae are well adapted to survival within phagocytic and non-phagocytic host cells (Poppiel and Turnbull, 1985), producing over 20 macrophage-induced proteins (MIPs) that provide protection within the hostile environment of the macrophage (Buchmeier and Heffron, 1990). These MIPs serve as immunogens and may be used to stimulate a humoral response by the host.

Salmonellae stimulate both a cellular and humoral immune response (Clarke and Gyles, 1993). In mammals, specific IgG, IgM, and IgA antibodies against salmonellae lipopolysaccharides have been identified. Specific IgA antibodies serve as the primary defense by binding to the salmonellae and preventing attachment to intestinal epithelial cells. Serum antibody levels do not correlate with protection against experimental challenge with live or killed *Salmonella* vaccines in calves (Lindberg and Robertsson et al., 1983). Immunization with an avirulent live vaccine produced no detectable serum antibody titer. These calves survived challenge with *S.* Dublin, compared to calves receiving inactivated vaccines that demonstrated detectable serum antibodies but developed clinical disease following challenge.

Salmonella vaccines are undergoing evaluation as a potential method to eliminate salmonellae in domestic animals. Inactivated and live attenuated vaccines are currently marketed. Regardless of the category, basic attributes of a vaccine include safety, not being pathogenic in either humans or animals, and efficacy, providing protection against intestinal colonization and organ invasion by multiple *Salmonella* serotypes. Suitable vaccines must be highly immunogenic, provide durable immunity, be genotypically stable, unaffected by either diet or host, and be simple to administer (Curtiss et al., 1993).

Inactivated vaccines are comprised of killed organisms or components of the organism. Inactivated vaccines have been used in domestic livestock with mixed results, with vaccine efficacy affected by immunogenicity and route of delivery. Inactivated vaccine strains that lose relevant antigens during vaccine production, or are subject to

elimination of select antigens by the host, do not stimulate an immune response (Barrow, 1991). Parenteral administration of inactivated vaccines may fail to elicit an appropriate cell-medicated response by T-lymphocytes. This is considered important to mucosal protection and limiting invasion by salmonellae (Collins, 1974).

Acetone and formalin are both used to inactivate salmonellae for vaccination. The protective ability of an acetone-inactivated *S.* Enteritidis oil emulsion vaccine was evaluated in laying hens (Gast et al., 1993). The vaccine was administered parenterally, twice at 4-week intervals, and then hens were challenged orally with 10^8 CFU of a field strain of *S.* Enteritidis. Incidence of intestinal colonization and fecal shedding were significantly lower in the vaccinated groups compared to the controls. More than 50% of the vaccinated chickens continued to shed *S.* Enteritidis. In a second study, a formalin-killed oil adjuvant *S.* Enteritidis vaccine administered by subcutaneous injection to chickens either at 3-weeks of age, or at both 3- and 6 weeks of age, protected the chickens against an intravenous or intramuscular challenge with 10^8 CFU of a field strain of *S.* Enteritidis (Timms et al., 1990). In these studies, inactivated vaccines reduced colonization with salmonellae, but did not result in absolute clearance of the pathogen.

Bacterial components, such as outer-membrane proteins (OMP), have been evaluated as potential candidates for vaccines. These proteins are highly antigenic and can stimulate both a humoral and cell-mediated immune response (Bouzoubaa et al., 1987). An OMP vaccine is considered to be safer than an attenuated vaccine because there is no possibility of virulence. A potential disadvantage associated with these

vaccines is that residual lipopolysaccharides in the OMP vaccines may initiate a hypersensitivity reaction in a susceptible host.

Administration of an adjuvant OMP from *S.* Enteritidis composed of either positively or negatively charged liposomes or lipid-conjugated immunostimulating complexes was tested in turkeys (Bouzoubaa et al., 1987). Subjects receiving the positively charged liposomal vaccine had a higher antibody titer than recipients of a killed bacterin. The OMP vaccines protected turkeys against organ invasion, with 90-100% of the tissue sample being *S.* Enteritidis negative. Effectiveness of an OMP vaccine has also been evaluated in chickens (Nagaraja and Rajashekara, 1991). Experimental birds were divided into three groups that received subcutaneous injections of either an *S.* Enteritidis OMP vaccine (N=30), a formalin-killed *S.* Enteritidis bacterin (N=30), or saline (non-vaccinated controls). Four weeks after the vaccination, one-half of the chickens in each treatment group were challenged with a field isolate of *S.* Enteritidis. Chickens vaccinated with the OMP protein and killed bacterin vaccines seroconverted during the study and titers increased after the field isolate challenge. One (6%) cloacal swab from an OMP vaccinated animal was *S.* Enteritidis positive compared to 9 (60%) of the non-vaccinated birds. Two (13%) of the tissue samples collected from the OMP and bacterin treatment groups were positive compared to 6 (46%) of the non-vaccinated controls. This study indicated that OMP vaccine can stimulate both humoral and cell-mediated protective responses.

Live attenuated vaccines can be administered orally to stimulate the natural invasion of the host cells, resulting in the production of secretory IgA on mucosal

surfaces, and the presentation of antigens to lymphocytes in gut-associated lymphoid tissues (Clark and Gyles, 1993). Intracellular pathogens, such as *Salmonella*, can mask themselves from the host immune system, becoming refractory to destruction and resulting in systemic dissemination in host cells, including macrophages. Live vaccines stimulate the host immune system to inhibit pathogenic salmonellae from using this system. Attenuated live vaccines are more effective in preventing clinical disease associated with intracellular pathogens because they stimulate a more intense cell-mediated response than inactivated vaccines.

Transposon mutagenesis (Maloy and Nunn, 1981), recombinant techniques that eliminate genes (Miller and Mekalanos, 1988), and culture-adapted organisms (Roof and Doitchinoff, 1995) have been used to develop avirulent attenuated *Salmonella* vaccines. The creation of a *gal*E mutant that is sensitive to galactose has been tested, but certain isolates, such as *S.* Cholerasuis and *S.* Typhi retain virulence (Nnalue and Stocker, 1986). The pho-P pho-Q regulatory system is responsible for regulation of genes that produce acid phosphatases and enable *Salmonella* to survive within macrophages (Miller et al., 1989). An attenuated *Salmonella* vaccine incorporating these mutants has been developed, but there is the potential for reversion to virulence (Miller and Mekalanos, 1990). The removal of the plasmid transposon and part of the gene responsible for invasion and replication within lymphoreticular tissue can be used to create an avirulent *Salmonella* vaccine (Barrow, 1991). Concern for reversion to virulence is a deterrent to commercial applications of these vaccines.

Swine challenged with a neutrophil-adapted *S.* Cholerasuis isolate were found to have significantly higher mean daily weight gain, lower body temperature and less severe clinical signs associated with salmonellosis following challenge with a virulent field strain of *S.* Cholerasuis (Kramer et al., 1987). The avirulent vaccine was safe for piglets as young as 3 weeks. Vaccination reduced organ invasion by the virulent field isolate and provided complete protection of the liver, colon and spleen.

Double-deletion mutations of *Salmonella* have gained favor because of the added safety associated with two gene deletions. In most cases, genes that are deleted are associated with metabolites required for survival. Avirulent vaccines that block the pathway for organisms that require para-aminobenzoate and 2,3-dihydroxybenzoate have been successfully generated for *S.* Cholerasuis, *S.* Dublin, *S.* Enteritidis, and *S.* Typhimurium (Hoiseth et al., 1981; Jones et al., 1991; Curtiss et al., 1993). Deletion (Δ) of the genes for adenylate cyclase (cya) and the cAMP-receptor protein (crp) affects regulation of up to 200 additional genes, many of which are responsible for the excretion of catobolites (Curtiss and Kelly, 1987; Clarke and Gyles, 1993). These attenuated vaccines stimulate humoral and cell-mediated immune responses with a low probability of reversion.

The avirulent, genetically modified Δ*cya* and Δ*crp S.* Typhimurium vaccine strain χ 3985 is a derivative of *S.* Typhimurium strain χ 3761, which is highly virulent to chickens (Hassan and Curtiss, 1994). Oral administration of *S.* Typhimurium strain χ 3985 to specific-pathogen free Leghorn chickens at 1 and 14 days of age provided protection against *Salmonella* Group B invasion of the spleen, ovary, bursa of

Fabricius, and also colonization of the ileum and cecum. Chickens challenged with heterologous strains, including *Salmonella* groups C,D, and E, were also protected against invasion of the spleen and ovary. Heterologous strains of *Salmonella* colonized the cecum and ileum of vaccinated birds, but at a reduced level compared to unvaccinated controls. Chickens vaccinated at 2 and 4 weeks of age were also protected against organ invasion and colonization of the ileum and cecum with *Salmonella* Groups B, D, and E. Vaccinated chickens challenged with *Salmonella* Group C showed some intestinal colonization and organ invasion, but at a lower intensity than unvaccinated chickens.

Chickens are susceptible to *Salmonella* at the time of hatch because of the immature state of their immune system (Hassan and Curtiss, 1990). Chickens challenged at day-of-hatch with a wild strain of *S.* Typhimurium develop transient lymphopenia, increasing the risk of opportunistic infections and the establishment of a *Salmonella* carrier status (Hassan and Curtis, 1994). Vaccination of hens with avirulent *S.* Typhimurium vaccine strain χ 3985 resulted in the presence of IgG antibodies in the egg yolk. Maternal antibodies are detectable as early as 3-days of age and decreased thereafter. Presence of maternal antibodies was correlated with protection against colonization of the progeny following experimental challenge (Hassan and Curtiss, 1996). Vaccination of progeny of immunized hens at 1- and 3-weeks of age does not provide the same protection against *Salmonella* colonization and organ invasion when compared to progeny vaccinated at 2- and 4-weeks of age. The maternal antibody in these chicks most probably reduced vaccine efficacy (Hassan and Curtiss, 1994).

Combining the vaccine with strict biosecurity could reduce *Salmonella* contamination of commercial poultry and reduce the incidence of food-borne salmonellosis in consumers of eggs and poultry meat.

2.9 Hypotheses

2.9.1 Epidemiologic Study of *Salmonella* in Green Iguanas on a Commercial Farm in El Salvador

The specific research objective of this study was to describe some of the epidemiology of *Salmonella* in captive green iguanas with special reference to commercial breeding and export to the USA. The associations of season, gender, and age class with *Salmonella* status were estimated. The primary hypotheses tested in this study were that adult iguanas are more likely to be *Salmonella*-positive than yearling iguanas, male iguanas are more likely to be *Salmonella* -positive than female iguanas, and that iguanas sampled in the spring are more likely to be *Salmonella*-positive than those sampled in the fall.

2.9.2 Sensitivity and Specificity Estimation of Three Diagnostic Tests for *Salmonella* in Green Iguanas

Objectives of this study were to estimate the prevalence of *Salmonella* in two populations of hatchling iguanas scheduled for export to the United States, and to estimate the sensitivity and specificity of a polymerase chain reaction assay, an enzyme-linked immunosorbent assay, and microbiologic culture for fecal *Salmonella* with the Bayesian estimation technique. Specific hypotheses tested in this study were that sensitivity of PCR assay would be higher than ELISA and microbiological culture, the

specificity of microbiological culture would be higher than PCR assay and ELISA, and the prevalence of *Salmonella* would be higher in iguanas after being transported.

2.9.3 Establishing a *Salmonella* Clearance Model With Enrofloxacin

Objectives of this study were to evaluate enrofloxacin as a method to suppress or eliminate *Salmonella* in immature green iguanas in a controlled environment and to develop a *Salmonella* clearance model to evaluate possible methods of control. The primary hypothesis evaluated in this study was that green iguanas treated with enrofloxacin would be less likely to be *Salmonella*-positive than iguanas treated with saline (H_0: $\mu_B=\mu_S$; H_1: $\mu_B<\mu_S$). The secondary hypothesis evaluated was that *Salmonella* would be detected at a higher frequency in necropsy specimens than cloacal swabs (H_0: $\mu_C=\mu_N$; H_1: $\mu_C<\mu_N$)

2.9.4 Evaluation of the Infectivity of *Salmonella* Typhimurium Strain 524 in Iguanas Following Enrofloxacin Elimination of *Salmonella*

Objectives of this study were to determine if immature iguanas could be infected with an oral inoculum of *Salmonella* Typhimurium strain 524, after treatment with enrofloxacin, to create an infection model that could be used to evaluate methods to eradicate Salmonella, and ascertain the long term effects of administering enrofloxacin on eliminating *Salmonella* colonization of the intestinal tract in the iguana. The primary hypothesis evaluated in this study was that green iguanas infected with *Salmonella* Typhimurium strain 524 after enrofloxacin treatment would be more likely to be *Salmonella*-positive than iguanas treated with saline (H_0: $\mu_I=\mu_S$; H_1: $\mu_I>\mu_S$). The secondary hypothesis evaluated in this study was that *Salmonella* would be detected

with a higher frequency from necropsy specimens than cloacal swabs (H_0: $\mu_C=\mu_N$; H_1: $\mu_C < \mu_N$).

2.9.5 Effect of an Avirulent *Salmonella* Vaccine on the Colonization of *Salmonella* in Green Iguanas

The specific research objective of this study was to evaluate a live attenuated mutant *Salmonella* vaccine as a method to eliminate *Salmonella* infection in immature green iguanas. The primary hypothesis evaluated in this study was that iguanas vaccinated with a commercial vaccine and infected with *Salmonella* Typhimurium strain 524 would be less likely to be *Salmonella*-positive than unvaccinated iguanas infected with the same strain of *Salmonella* (H_0: $\mu_I=\mu_S$; H_1: $\mu_I>\mu_S$). A secondary hypothesis was evaluated to determine if *Salmonella* would be detected at a higher frequency from necropsy specimens than cloacal swabs (H_0: $\mu_C=\mu_N$; H_1: $\mu_C<\mu_N$).

CHAPTER 3

MATERIALS AND METHODS

3.1 Epidemiologic Study of *Salmonella* in Green Iguanas on a Commercial Farm in El Salvador

The incidence of reptile-associated salmonellosis in humans has increased dramatically during the 1990's (Wong et al., 2000). Green iguanas have been identified as a reservoir of *Salmonella* and have been implicated in a number of salmonellosis cases in humans (Centers for Disease Control, 1995). No study has been performed to determine the epidemiology of *Salmonella* in green iguanas. Two cross-sectional studies were performed in November 1999 and March 2000 at the largest commercial iguana farm in El Salvador to estimate prevalence of *Salmonella* in iguanas and their environment and to determine whether age class, gender or season have an effect on *Salmonella* prevalence in the iguanas or their environment.

3.1.1 Iguana Farm Description

The farm produces over 200,000 iguana hatchlings annually. The facility comprised 21 pens over a combined area of 8 Ha. The pens ranged in size from 184 m^2 to 4,955 m^2, and are surrounded with galvanized corrugated iron sheeting approximately 150 cm above ground. Pens that housed hatchling iguanas were covered with nylon netting to prevent predation. The breeding colony comprised approximately 29,000 females and 12,000 males. Shelter was provided by concrete-block and bamboo-roofed structures. Fecal material was removed manually from the pens and incinerated at unspecified intervals.

Non-chlorinated drinking water was supplied to the iguanas in cement basins located in each pen and was replaced every 72 hours. Water basins were disinfected with a combination of iodine (11.2 g/100ml) and phosphoric acid (2.0 g/100ml) before being refilled. Iguanas were fed daily with a diet that included rice, wheat, fresh sorghum, spinach, and carrots. Electrolyte, vitamin, amino acid, and mineral supplements were added to the feed daily. Vegetables were stored in an open shelter surrounded by a 1.2-m fence. The diet was mixed in a commercial gasoline-powered grinder to produce a diet with a homogenous coarse consistency. Food was deposited from the grinder on to the concrete floor of the preparation area. Shovels were used to transfer feed into wheel barrows, which were used for transport to the pens. Feed was placed on plastic trays in the pens. The surfaces of the food grinder, food preparation area, and food trays were disinfected with 10% chlorine bleach daily.

3.1.2 Fall 1999 Cross-Sectional Study

From November 1-4, 1999, a cross-sectional study was performed at the iguana farm in El Salvador, with specimens collected from both adult and 7- to 8-month-old iguanas, feed and the environment. Samples were obtained from the feed ingredients, the commercial grinder, mixed feed at the preparation site, and hands of the food preparers. Water samples were collected from the well and every filled water basin in use in the pens. Environmental specimens included soil collected from the pens, insects, and free-living lizards captured on the farm.

Three-hundred two adult male iguanas and 405 adult female iguanas were collected by hand from the largest breeding pen (4,955 m^2) on the farm. Male and

female iguanas were well-mixed within the pen, and copulation was observed during sample collection. Five farm employees entered the pens and collected iguanas by hand as they fanned out across the pen from their point of entry. The impracticality of enumerating the iguanas in the pen and the risk of injury to the animals associated with capture, precluded a more rigorous selection method. Once captured, animals were restrained by hand for sampling. A cloacal swab was collected with a sterile cotton-tip applicator. The swab was inserted approximately 2 cm into the cloaca, traversing the proctodeum and coprodeum, into the colon and was then rotated 5 times. The cotton-tip applicator was immediately placed into 1-ml of Stuart's transport media and stored in a wet ice-filled styrofoam cooler. Specimens were identified by number (1-302 M; 1-405 F). Upon completion of the sample collection, swabs were transferred to a refrigerator. One-hundred twenty 6- to 7-month-old iguanas out of approximately 2,000 yearling iguanas were collected from a single pen (675 m^2) and sampled with the previously described techniques, although only three farm employees were used to collect the iguanas. All swabs were stored on wet ice and transported by air to the LSU-SVM within 24 hours of collection. Upon arrival to the LSU-SVM, swabs were transferred by a technician into 7-ml of selenite enrichment broth and incubated at 37°C under aerobic conditions for 24 hours.

Twenty-five samples of the rice, wheat, and mineral supplement were collected from unopened 50-kg sacks as delivered from a commercial feed mill. Twenty samples of the electrolyte, amino acid and vitamin supplement were collected from unopened 100 g packets as delivered from the manufacturer. Once the bags were opened, a 20g

sample was collected off of the surface of the food product by aseptic technique and placed into a sterile Whirl-pak ® bag (Nasco, Cedarsburg, Wisconsin, USA). Ten representative mixed feed samples (20 g) were collected by aseptic technique for two consecutive days and placed into sterile Whirl-pak ® bags. Sterile cotton-tip applicators were used to collect six samples on three consecutive days from the cutting surfaces of the food grinder. Swabs were placed into Stuart's transport media, refrigerated, and shipped on wet ice to the LSU-SVM within 24 hours of collection; samples in Whirl-Pak ® bags were transported at ambient temperature. Swabs were processed as previously described.

Soil samples were collected from each pen on the farm. A simple random selection technique was used to collect a soil sample from every 150 m^2. Four quadrants within each 150 m^2 were designated A through D. A soil sample was collected from a quadrant selected by random drawing. A minimum of two samples were collected from the smallest pens. Approximately 20 g of soil was collected from each site with a sterile tongue depressor and was placed into a sterile Whirl-Pak ® bag. The samples were transported under ambient temperature by air to the LSU-SVM.

Water samples were collected from both an active and a stagnant well, and from all of the water basins in use in each pen. Well samples were collected on three consecutive days and one water sample was collected from the drain of each water basin. Water in the basins had not been changed within the 24 hours preceding the sample collection. Water was collected into sterile plastic Whirl-Pak® bags and transported at ambient temperature to the LSU-SVM.

Three 26.4-cm x 52.8-cm sheets of commercial fly paper were placed at the food preparation site on two consecutive nights to trap insects. A sterile cotton-tip applicator was used to crush one-hundred flies trapped on the paper. The swabs were placed into Stuart's transport media and refrigerated. Samples were transported to the LSU-SVM on wet ice by air within 24 hours of collection to the LSU-SVM and processed as previously described.

Farm workers captured 18 green iguanas (wild type), nine amevias (*Amevia* spp.), one skink (*Cnemidophorus lemniscatus*), and one basilisk (*Basiliscus basiliscus*) on the farm premises. A sterile cotton-tip applicator was used to collect cloacal cultures from these animals with the techniques previously described. Swabs were placed into 1-ml of Stuart's transport media and refrigerated. Samples were transported to the LSU-SVM on wet ice by air within 24 hours of collection. Swabs were transported to the LSU-SVM and processed as previously described.

After the conclusion of the sampling in El Salvador, all of the environmental and feed samples stored in Whirl-Pak® bags were transferred into 7-ml of selenite enrichment broth and incubated at 37°C for 24 hours under aerobic conditions at the LSU-SVM. After incubation, the enriched selenite cultures were mixed on a Vortex agitator for 5 seconds. A heat-sterilized bacterial loop was used to transfer an aliquot of enriched broth to the surface of a petri dish containing xylose-lysine-tergitol agar (XLT-4) (Remel, Lenexa, KS). Streaked plates were incubated at 37°C for 24 hours under aerobic conditions. Presumptive *Salmonella* colonies were evaluated on indicator media including urea, lysine iron agar (LIA), and triple iron agar (TSI). Negative samples were

retested after a 48-hour delayed secondary enrichment. A heat-sterilized bacterial loop was used to streak a portion of a suspect colony onto slants of urea, LIA and TSI agar, and preparations were incubated aerobically at 37°C for 24 hours. The presence of *Salmonella* was denoted by a negative urea test, positive LIA with hydrogen sulfide production (H_2S), and alkaline over acid with H_2S in the TSI. Presumptive *Salmonella* colonies were further evaluated with API 20E Test Strips® (bioMerieux Vitek, Inc., Hazelwood, MO). A heat-sterilized bacterial loop was used to transfer sample colonies from the TSI slant to 10 ml of 0.85% saline to attain a concentration equivalent to a 0.5 McFarland's equivalence turbidity standard (Remel, Lenexa, KS). Samples were placed into designated receptacles on the test strips in accordance with the manufacturer's directions and incubated aerobically at 37°C for 24 hours. Bacterial reactions were interpreted with the appropriate key compiled by the manufacturer of the test strip.

3.1.3 Spring 2000 Cross-Sectional Study

From March 27-30, 2000, specimens were collected from adult iguanas, yearling iguanas, hatchlings, embryos, egg surfaces, feed, and environment. Feed and environmental samples were obtained with the previously described techniques.

Three-hundred eighty adult male iguanas and 407 adult female iguanas were collected by hand from large holding pens on the farm by the same method used in the previous cross-sectional study. Adult male iguanas were all captured from a single pen (4,532 m^2) that housed approximately 12,000 male iguanas. Adult females were captured from two different pens (IT- 2: 4,980 m^2; IT-3: 4,480 m^2), each housing approximately 8,000 adult female iguanas. Cloacal swabs were obtained, labeled,

stored, and shipped as described earlier. Three-hundred yearling green iguanas were collected from three different holding pens (MB-1: 185 m^2, MC-1: 366 m^2, MD-3: 577 m^2) on the farm by the same method described for the adults, although only three farm employees were used to collect the iguanas. There were approximately 2,000 iguanas in pen MB-1, 3,500 iguanas in pen MC-1, and 6,000 iguanas in pen MD-3. Cloacal swabs were obtained, labeled, stored and shipped as described previously.

Specimens of ovary, oviduct, liver, small intestine, and colon, were collected from adult female iguanas for microbiological examination. Twenty-five adult female iguanas were collected from a pen (MC-2: 366 m^2) holding approximately 1,000 adult female iguanas by three farm employees with the techniques described earlier. The iguanas were euthanized by cervical dislocation, and specimens were collected at post-mortem examination within 1 hour by sterile techniques. The ventral surface of the iguana was decontaminated with 70% isopropyl alcohol and flamed to carbonize organic debris. A paramedian incision was made into the coelomic cavity with a #15 scalpel blade. The left ovary, left oviduct, liver and gall bladder (1 gr sample), entire small intestine, and colon (2 gr) were excised by sterile techniques, and tissue samples were placed into individually numbered tubes with 7-ml of selenite enrichment broth. Necropsy instruments were swabbed with sterile cotton-tip applicators, which were then placed into 1-ml of Stuart's transport media. Samples were stored on wet ice, transported to the LSU-SVM, and processed by the techniques described previously.

Specimens of the internalized yolk, liver, small intestine, and colon were collected from hatchling iguanas. Thirty-five hatchling iguanas collected from pen M-5

(4,955 m^2) were euthanized by cervical dislocation with specimens collected at post-mortem examination within 1 hour by sterile technique. There were approximately 200 hatchling iguanas in the pen at the time of collection. The ventral surface of the iguanas were decontaminated with 70% isopropyl alcohol and flamed to carbonize organic debris. A paramedian incision was made into the coelomic cavity with a #15 scalpel blade. An incision was made into the internal yolk sac with a sterile #15 scalpel blade. A Mini-tip culturette® was introduced into the yolk sac to obtain a representative specimen. The entire liver and small intestine-colon of the hatchling were excised by sterile technique and placed into individually numbered tubes with 7-ml of selenite enrichment broth. Necropsy instruments were swabbed with sterile cotton-tip applicators which were then placed into 1-ml of Stuart's transport media. Samples were stored on wet ice , transported to the LSU-SVM, and processed with the techniques described previously.

Eight iguana nests were excavated on the farm to determine the prevalence of *Salmonella* on the egg shell surface, the embryos, and in the nest soil. Nest selection was based on random excavation of areas within the pens (M-4, M-5, M-1, and M-2) that were considered potential nest sites. Three nests were excavated in M-4 to depths of 68-cm (M-4 nest 1), 78-cm (M-4 nest 2), and 53-cm (M-4 nest 3). One nest was excavated in M-5 to a depth of 42-cm (M-5 nest 1). Two nests were excavated in M-1, both to a depth of 75-cm (M-1 nest one and M-1 nest 2). In M-2, two nests were excavated to depths of 48-cm (M-2 nest 1) and 53-cm (M-2 nest 2). Six eggs were sampled from each nest in M-5, M-1, and M-2, and 12 eggs were sampled from each

nest in M-4. The external surface of each egg was swabbed with a sterile cotton-tip applicator, which was immediately transferred to a tube containing 1-ml of Stuart's transport media. One side of the egg was decontaminated with 70% isopropyl alcohol, which was allowed to dry, and the area was incised. Embryos were fully developed within the eggs, but the yolk-sacs had not been internalized. The embryonic yolk sac was swabbed with a sterile cotton-tip applicator and the swab was placed into 1-ml of Stuart's transport media. Embryos were removed from their eggs, euthanized, macerated and placed into 7-ml of selenite enrichment broth. Swabs were transported on wet ice to the LSU-SVM and processed as previously described.

Six nest soil samples were collected from M-4 nest 1, M-1 nests 1 and 2, M-2 nests 1 and 2, and M-5 nest 1. An additional six soil samples were collected within the walls of each excavated nests. Samples were placed into sterile Whirl-pak® bags and transported at ambient temperature to LSU-SVM for processing.

Twenty-five samples of the rice, wheat, and mineral supplement were collected and tested with the previously described methods. Specimens of the mixed food samples, grinder and food preparation site, and swabs from the food preparers hands were also collected.

Soil samples were collected from each pen on the farm. A simple random selection technique was used to collect a soil sample from every 150 m^2. Four quadrants within each 150 m^2 were designated A through D. A soil sample was collected from a quadrant selected by random drawing. A minimum of two samples were collected from the smallest pens. Water samples were also collected and

processed as described previously. One-half of the shelters in the pens were assayed for the presence of *Salmonella*, including four in MD-1, seventeen in M-4, seven in M-5, two in MA-1, two in MB-1, three in MC-1, ten in M-1, six in MA-2, ten in MB-2, eight in M-2, three in MD-2, two in MC-2, twelve in MD-3, five in M-3, six in MA-3, seven in MB-3, and six in MC-3. A sterile cotton-tip applicator was rolled on the surface of the shelter and placed into 1-ml Stuart's transport media. The samples were processed with the techniques described earlier.

Farm workers captured seven wild lizards on the farm, including three green iguanas (wild type), three skinks (*Cnemidophorus lemniscatus*), and one basilisk (*Basiliscus basiliscus*). Cloacal-colon swabs were collected from each of the animals and samples processed following the techniques described previously. Fly paper was placed around the feed preparation area to trap insects. The fly paper was examined twelve hours after being placed and specimens were processed as described previously.

At the time of this study, there were very few hatchling iguanas available for testing. Approximately 1-month after the study, April 25, 2000, cloacal swabs were collected from 300 hatchling iguanas captured by hand by farm employees from pen M-4 (4,950 m^2). The owner of the farm collected the samples using a Mini-tip culturette®. The samples were transported on ice by air to the LSU-SVM within 24 hours of collection for further processing.

Upon arrival at the LSU-SVM, environmental samples were transferred into 7 ml of selenite enrichment broth and incubated at 37°C for 24 hours under aerobic conditions. After incubation, the enriched selenite cultures were mixed on a Vortex

agitator for 5 seconds. A heat-sterilized bacterial loop was used to transfer an aliquot of enriched broth to the surface of a petri dish containing xylose-lysine-tergitol agar (XLT-4) (Remel, Lenexa, KS). Microbiological samples were processed with the previously described techniques.

3.1.4 Statistical Analyses

The 95% binomial confidence intervals (CI) were calculated for each of the prevalence estimates (Hassard, 1991). In cases where the prevalence estimate was 0, the 95% confidence intervals were calculated with the technique described by van Belle and Millard (1998). True prevalence estimates were calculated for each of the apparent prevalence estimates using the Bayesian estimates for sensitivity and specificity for microbiological culture. Odds ratios were calculated to quantify the association of season (Fall, Spring), gender (Male, Female) and age class (Adult, Yearling) on microbiological outcome. The association between microbiological outcome and age class was further evaluated while controlling for gender. Stratum-specific OR were calculated for age class, while controlling for gender. Testing for homogeneity of stratum-specific odds ratios was performed with the Breslow and Day (BD) test statistic (Sahai and Khurshid, 1996). In the absence of effect modification, Mantel-Haenszel weighted odds ratios (MH OR) (Sahai and Khurshid, 1996) were compared with crude OR to test gender as a potential confounder. If the difference between the crude OR and the MH OR was greater than 10%, then gender was considered a confounder. Associations between microbiological outcome and age class and microbiological outcome and gender were further evaluated while controlling for season with the

previously described techniques. Soil sample and water sample results between the two sampling periods were compared with a Pearson's χ^2 test for homogeneity. Values of $p < 0.05$ were considered statistically different. Statistical analysis was performed with EpiInfo 2000 (Centers for Disease Control, Atlanta, GA, 2000) and StatExact 3 (Cytel Software, Corp., Cambridge, MA).

3.2 Sensitivity and Specificity Estimation of Three Diagnostic Tests for *Salmonella* in Green Iguanas

Bayesian methods were used to estimate the prevalence of *Salmonella* in two different populations of iguanas exported to the United States, and to estimate the sensitivity and specificity of a PCR assay, ELISA and microbiological culture. Three assays were applied to two separate populations, and the latent frequency of infected animals estimated for each.

3.2.1 Description of Populations Sampled and Sample Collection

Iguanas were collected from two genetically distinct and geographically separated commercial farms in Costa Del Sol, El Salvador. Farm 1 produced over 200,000 hatchling green iguanas in 1998, while Farm 2 produced approximately 50,000 hatchlings during the same year. One-hundred and nineteen, 4-5 month old captive-born, green iguanas were acquired from each farm. The iguanas tested were the cohort of animals being shipped to the United States on September 5, 1999 and represented the age class of iguanas being purchased for the pet trade. Farm employees collected the test iguanas by hand from a holding pen and placed them into individual nylon bags.

Farm workers carried the iguanas to the processing area and placed them into shipping boxes. A cloacal swab was obtained from each iguana with a Mini-tip culturette® (Becton Dickinson and Company, Cockeysville, MD, USA) prior to being placed into the shipping boxes. Pre-shipment cloacal swabs were collected by the farm owner based on a specified protocol. The swab was inserted approximately 2 cm into the cloaca, traversing the proctodeum and coprodeum, into the colon and was then rotated 5 times. This specimen was identified by number and was designated sample "A". Culturettes were stored on wet ice and transported by air to the Louisiana State University, School of Veterinary Medicine (LSU-SVM) within 24 hours of collection. After the cloacal swabs were collected, iguanas were placed into individually numbered, ventilated plastic containers, transported to the El Salvador international airport, and sent by air the next day to the LSU-SVM. The iguanas from Farms 1 and 2 arrived at the LSU-SVM within 48 and 96 hours of shipment, respectively. Upon arrival at the LSU-SVM, a second cloacal swab (sample "B") was collected by the author using the previously described technique.

Culturettes were placed in 7-ml of selenite enrichment broth, incubated at 37°C for 24 hours under aerobic conditions, mixed on a Vortex apparatus for 5 seconds, and divided among three aliquots of approximately 2-ml. The "A" samples were assayed for *Salmonella* by both microbiological culture and PCR and the "B" samples were assayed with culture, ELISA and PCR.

3.2.2 Microbiological Culture

A heat-sterilized bacterial loop was used to transfer an aliquot of enriched broth to the surface of a petri dish containing xylose-lysine-desoxycholate agar (XLD)(Remel, Lenexa, KS). Streaked plates were incubated at 37°C for 24 hours under aerobic conditions, and presumptive *Salmonella* colonies were evaluated with the previously described techniques.

3.2.3 Enzyme-Linked Immunosorbent Assay

A commercial ELISA test (Salmonella ELISA Test 96/1, Bioline, Polarvej 60, DK-7100 Vejle, Denmark) licensed for *in vitro* determination of *Salmonella* from human enteric samples, was used for this study. This commercial test incorporates purified rabbit antibodies specific to *Salmonella* flagellar antigens as the substrate to bind the *Salmonella* proteins, and purified enzyme-conjugated goat antibodies as the marker.

The 2-ml selenite aliquots were boiled for 20 minutes in a closed test tube and then cooled at room temperature for one hour. Test strips were allowed to warm to room temperature while the selenite samples were cooled. The first well of the test strip was used as a blank and the second and third wells represented the negative and positive controls, respectively. The negative control contained Mannose-broth with stabilizer and the positive control contained heat-killed *Salmonella* Typhimurium (10^6 bacteria/ml) in Mannose-broth with stabilizer. The selenite samples were pipetted into their respective wells, with the last two wells used as secondary negative and positive controls. The test strips were covered with foil and incubated aerobically at 37°C for 30 minutes. After

incubation, the wells were emptied and washed five times with a commercial buffer compound, composed of 0.075 M Tris-HCl/2.5 M Na Cl and 5% Tween 20. A horseradish peroxidase-antibody conjugate was then added to all of the wells except the blank, and test strips were incubated aerobically at 37°C for 30 minutes. A second rinse was performed with the commercial buffer. The blue colored substrate, composed of 3,3',5,5'-tetramethylbenzidine and 0.03% hydrogen peroxide, was added to all of the wells and the samples were incubated for 15 minutes at room temperature. An aliquot of 0.2 M sulphuric acid was added as a stop solution to all of the wells and the reaction was read within 10 minutes. A yellow color change within the well indicated a positive reaction. The optical density of the test wells was quantified with a photometer fitted with a 450 nm filter. An optical density reading greater then 0.200 was considered positive.

3.2.4 Polymerase Chain Reaction

The selenite samples for the PCR assay were stored on wet ice and transported by air to the University of Georgia, College of Veterinary Medicine, Athens, GA. Extraction of the *Salmonella* deoxyribonucleic acids (DNA) was accomplished by a combination of centrifugation and heating. Samples were first centrifuged at 8,000 rpm for 5 minutes. The supernatant was removed and the pellet resuspended in 100µl of distilled water. Samples were then heated at 95°C for fifteen minutes, and then centrifuged at 10,000 rpm for 5 minutes. The supernate was removed and used for the DNA analysis.

The PCR assay was performed with an 11 µl sample comprised of 1 µl of the DNA extract and 10 µl of the following: 1X PCR buffer (50mM Tris-HCl, 250 µg/ml BSA, pH 8.3, 3 mM $MgCl_2$) (Idaho technologies, Salt lake City, UT), 200µM dNTPs, 0.5µM of the primers, 0.4 U/µl *Taq* polymerase, 0.056 µM TaqStart Antibody (Clonetech Laboratories, Palo Alto, CA), 0.2 uM Fluorescein-labeled probe, and 0.4 uM Cy5-labeled probe or 0.4 uM LC Red-640-labeled probe. The primer sequences used for this study are proprietary to the University of Georgia Research Foundation, Athens, GA. The procedure comprised one cycle of 94°C for 5 seconds, followed by forty-five cycles of 94°C for 0 seconds, 52°C for 2 seconds, and 72°C for 10 seconds, and finally one additional cycle of 94°C for 0 seconds, 40°C for 15 seconds, and 70°C for 4 seconds. Confirmation of a positive sample was made by analyzing the amplitude and melting temperatures (*Salmonella*: 54-56°C) for the probes with software provided by the manufacturer. Thermal cycling procedures were performed with an Idaho Technology Rapid Cycler (Salt Lake City, UT).

3.2.5 Bayesian Methods: Estimation of Population Prevalence and Test Characteristics

As new diagnostic tests become available, there is a need to compare them to other standard tests, preferably a gold standard, to determine their sensitivity and specificity. Clinicians and public health officials rely on this information when applying specific health programs. Unfortunately, it is very difficult to evaluate new testing methods when there is no gold standard or when the cost of a gold standard is too great. Bayesian analysis can be used to estimate the sensitivity and specificity of one or more

tests in one or more populations in the absence of a gold standard (Joseph et al., 1995). Unlike non-Bayesian methods that may be hindered by the constraints of having more parameters to estimate than degrees of freedom, Bayesian methods eliminate the need for constraints by using prior distributions for the parameters being tested. Prior distributions are estimated for the sensitivity and specificity of each assay and the prevalence(s) of the population(s) with the best available information. The prior distributions are combined with the observed data, through the likelihood function, to derive posterior distributions through the use of a Gibbs sampler. The posterior densities are the product of independent beta errors for each parameter and provide updated beliefs about the test characteristics that can be used to calculate median sensitivities, specificities, prevalences, and 95% credible intervals.

Estimates of the prior error distributions for the sensitivity and specificity of each assay were obtained from the literature (Cohen et al., 1996; Pelton et al., 1994; Tan et al., 1997) and expert opinion. The prior point estimates selected for the Bayesian estimation were a PCR sensitivity of 90% and specificity of 95%, an ELISA sensitivity of 80% and a specificity of 90%, and microbiological culture sensitivity of 70% and specificity of 99%. Prior-point estimates for the prevalences in each population were taken to be the proportion of PCR positive animals from each farm. The range of values representing uncertainty in the PCR sensitivity was ± 0.10 and ±0.05 for the specificity, the ELISA sensitivity was ± 0.10 and ±0.10 for the specificity, and the microbiological culture was ± 0.10 for the sensitivity and ±0.01 for the specificity.

A prior beta density was calculated for each test parameter by matching the starting value of the point estimate to the mean of the beta distribution ($\alpha/(\alpha+\beta)$) and matching one quarter of the total range with the standard deviation ($((\alpha\beta/((\alpha+\beta)^2(\alpha+\beta+1)))^{1/2}$) of the beta distribution (Joseph et al., 1995). The values represented by α and β are the parameters of the β distribution fitted to each prior. Beta distributions are used because they give probability 1 to a finite interval (0,1), matching the range of all parameters of interest in this study. The prior distributions and observed data were simulated using the Gibbs sampler. The posterior densities derived from the Gibbs sampler were used to calculate the median sensitivities and specificities for each assay, the prevalences for each farm, and the associated 95% credible intervals.

Prevalence, and PCR and microbiological culture sensitivity and specificity, were estimated independently for each farm for the samples collected in El Salvador using the tt2 software designed by Joseph et al. (1995). The tt3 software designed by Joseph et al. (1995) was used to estimate the prevalence, and sensitivity and specificity for the PCR, ELISA, and microbiological culture assays, independently for each farm for the samples collected in Baton Rouge. After independent evaluation of the populations, the PCR and culture assays from both populations were evaluated simultaneously using a modification of the Joseph et al. (1995) software as described by Singer et al. (1998). The software was also used to evaluate PCR assay and ELISA, and ELISA and microbiological culture, for both populations for the Baton Rouge samples. Sensitivity analysis was performed to evaluate the robustness of the Bayesian analysis to misclassification. Data perturbations to observed cell counts were done and additional

Bayesian analysis performed. The posterior distributions of the prevalences and the test parameters derived from the different models were compared and evaluated for change. Analysis was performed using S-Plus software version 4 (MathSoft, Inc., Seattle, WA).

3.3 Establishing a *Salmonella* Clearance Model With Enrofloxacin

3.3.1 Study Design and Sample Collection

In September 1999, an intervention study was performed to determine if enrofloxacin could be used to eliminate *Salmonella* in young iguanas maintained in a *Salmonella*-free environment. Iguanas were acquired from the commercial farm described in section 3.1.1. One-hundred 4 to 5 month-old green iguanas were collected by the farm employees using the techniques described previously and transported by air to Fluker Farms, Port Allen, LA for *Salmonella* assay.

Cloacal swabs were obtained with a sterile cotton-tip applicator. The swab was introduced approximately 2 -cm into the cloaca, through the proctodeum and coprodeum, into the colon and rotated 5 times. The cotton-tip applicator was immediately placed into 7-ml of selenite enrichment broth and the samples were transported via automobile under ambient temperature to the LSU-SVM for further processing. The samples were individually numbered (1-100). Each iguana was placed into an individually numbered plastic enclosure by itself and housed at Fluker Farms until the samples were processed.

Upon arrival at the LSU-SVM, the selenite samples were incubated at 37°C for 24 hours under aerobic conditions. After incubation, the enriched selenite cultures were mixed on a Vortex agitator for 5 seconds. A heat-sterilized bacterial loop was used to

transfer an aliquot of enriched broth to the surface of a petri dish containing xylose-lysine-tergitol agar (XLT-4, Remel, Lenexa, KS, USA). The remainder of the selenite broth was refrigerated (40°F) until submitted to the University of Georgia for PCR testing at the completion of the study. Microbiological testing and PCR assay testing for *Salmonella* followed the protocols documented previously. Culture negative samples were retested after a 5-day delayed secondary enrichment. Parallel testing (PCR assay and microbiological culture) was used to increase the overall sensitivity of the *Salmonella* shedding detection.

An antimicrobial sensitivity profile was performed on confirmed *Salmonella* isolates. The *Salmonella* isolates were streaked onto a 5% sheep-blood agar plate and incubated at 37°C for 24 hours under aerobic conditions. Representative colonies were collected from the blood agar plate and placed into double-distilled water until the optical density of the solution was equal to a 0.5 McFarland's standard. A sterile cotton-tip applicator was used to collect a sample and to streak it on to a Mueller-Hinton agar plate. A 5 μg enrofloxacin-impregnated disc was then placed onto the agar plate. The samples were incubated at 37°C for 24 hours under aerobic conditions. The sensitivity of the organism was determined with a metric ruler. The organism was considered to be sensitive to enrofloxacin if the zone of inhibition surrounding the disk was greater than 17 mm.

Forty iguanas that were identified as being *Salmonella*-positive with an enrofloxacin sensitive isolate were transferred from Fluker Farms to the LSU Life Sciences Vivarium. A physical examination was performed on each animal and the

weight was recorded. The iguanas were divided into two groups of twenty and housed in two separate rooms in Rubbermaid® plastic containers (24" x 18"x 12"). Holes were drilled into the sides of the container for ventilation and a screen panel placed in the cover to improve airflow. Newspaper sheets were placed in the bottom of the container as a substrate. The container was replaced with a sterilized unit daily to reduce accumulation of contamination. The iguanas were fed a commercial iguana chow that was free of *Salmonella* according to microbiologic culture. Water and food were placed into ceramic bowls that were changed daily. Room temperature was maintained between 30 and 32°C. Biosecurity precautions followed by caretakers to prevent the spread of *Salmonella* between the two treatment rooms included putting on new disposable gloves, mask, and boot covers on entry to each room. A sodium hypochlorite footbath was also positioned outside each room.

The iguanas were divided into two groups by random drawing, designated the "enrofloxacin treatment group" (Group 1) and a "saline treatment control" (Group 2). Iguanas in the enrofloxacin treatment group received 10 mg/kg enrofloxacin suspension (Bayer, Shawnee Mission, MO) *per os* daily for fourteen days. Iguanas in the saline treatment control group received an equal volume of 0.9% sterile saline *per os* daily for fourteen days.

A sterile cotton-tip applicator was used to collect a cloacal-colon culture from each iguana on the first, seventh and fourteenth day after the enrofloxacin or saline treatment. The culture swab was placed into 7-ml of selenite enrichment broth and

incubated at 37°C for 24 hours under aerobic conditions. Microbiological testing and PCR assay for *Salmonella* followed the protocols documented previously.

Iguanas were humanely euthanized fifteen days after the completion of the 14-day enrofloxacin or saline treatment. Ketamine hydrochloride (200 mg/kg) was administered intramuscularly in the anconeus muscle. A 3/4 inch, 25 gauge needle fastened to a 1-ml syringe was used to collect an intracardiac (ICa) blood sample. The 1-ml blood sample was immediately placed into 7-ml of selenite enrichment broth and incubated at 37°C for 24 hours under aerobic conditions. After the blood sample was collected, Beuthanasia solution (0.2 ml/100 g) (Schering-Plough, Union City, NJ) was administered by the intracardiac route for euthanasia.

Iguanas treated with enrofloxacin were processed first to reduce the risk of cross-contamination. The ventral surface of the iguanas was decontaminated with 70% isopropyl alcohol and flamed to carbonize organic debris. A paramedian incision was made into the coelomic cavity with a #15 scalpel blade. The liver and gall bladder (1g), spleen, small intestine and colon (2 g) were excised by sterile techniques, and tissue samples were placed into individually numbered tubes with 7-ml of selenite broth. All of the tissue samples were macerated within the enrichment broth and incubated at 37°C for 24 hours under aerobic conditions. Attempts to isolate *Salmonella* with microbiologic culture and PCR assay were similar to those described previously.

3.3.2 Sample Size Determination

Sample size was calculated for a binomial proportion under the following assumptions and criteria: That the proportion of *Salmonella*-positive iguanas in the

enrofloxacin treatment group would be no greater than 0.3 after treatment, that the proportion of *Salmonella*-positive iguanas in the saline treatment group was no less than 0.8, that the $\alpha = 0.05$, and the power = 0.85 (Martin et al., 1987).

3.3.3 Statistical Analyses

The 95% binomial confidence intervals were calculated for the proportion estimates of the different treatment groups and sample specimens (cloacal swabs and necropsy specimens). In cases where the prevalence estimate was 0, the 95% confidence intervals were calculated with the technique described by van Belle and Millard (1998). The primary hypothesis evaluated in this study was that green iguanas treated with enrofloxacin would be less likely to be *Salmonella*-positive than iguanas treated with saline (H_0: $\mu_B=\mu_S$; H_1: $\mu_B<\mu_S$). Treatment groups (enrofloxacin vs. saline) were compared with the χ^2 test for homogeneity. The secondary hypothesis was that *Salmonella* would be detected at a higher frequency in necropsy specimens than cloacal swabs (H_0: $\mu_C=\mu_N$; H_1: $\mu_C<\mu_N$). Comparison of the cloacal swabs and necropsy results for each individual treatment group was made with the Fisher's exact test because an expected cell value was < 5. Values of $p< 0.05$ were considered statistically different. Statistical analysis was performed with EpiInfo 2000 (χ^2 test)(Centers for Disease Control, 2000) and StatExact 3 (Fisher's exact test)(Cytel Software, Cambridge, MA).

3.4 Evaluation of the Infectivity of *Salmonella* Typhimurium Strain 524 in Iguanas Following Enrofloxacin Elimination of *Salmonella*

3.4.1 Study Design and Sample Collection

In March 2000, an intervention study was performed to the infectivity of *Salmonella* Typhimurium strain 524 to iguanas after enrofloxacin treatment. The iguanas were acquired from the commercial farm described in section 3.1.1. One-hundred 9- to 10-month-old green iguanas were collected by the farm employees and transported by air to Fluker Farms, Port Allen, LA for *Salmonella* assay. Cloacal swabs were collected and processed with the previously documented techniques. The iguanas were housed at Fluker Farms until the samples were processed.

Microbiologic culture and PCR assay techniques were similar to those described previously. Parallel testing, with microbiological isolation and PCR assay, was done as previously described. Culture negative samples were retested after a 5-day delayed secondary enrichment. An antimicrobial sensitivity profile was performed for each isolate as previously described. However, a nalidixic acid sensitivity profile was performed in addition to the enrofloxacin profile. A 50-μg nalidixic acid disk was used for the sensitivity profile. Sensitivity to nalidixic acid was classified as a zone of inhibition greater than 20 mm.

Thirty-eight iguanas identified as being *Salmonella*-positive with enrofloxacin and nalidixic acid sensitive isolates, were transferred from Fluker Farms to the LSU Life Sciences Vivarium. A physical examination was performed on each animal and the

weight recorded. The iguanas were divided into groups of 18 and housed in separate rooms. Iguanas were maintained under the same conditions as described previously.

The green iguanas were divided by random drawing between an "infected treatment" group (Group 1) and a "saline treatment" group (Group 2). All 38 iguanas were given 10 mg/kg enrofloxacin suspension (Bayer, Shawnee Mission, MO) *per os* daily for fourteen days. A sterile cotton-tip applicator was used to collect a cloacal-colon culture from each iguana on the first, seventh and fourteenth day after the completion of the enrofloxacin treatment. The culture swab was immediately placed into 7-ml of selenite enrichment broth and incubated at 37°C for 24 hours under aerobic conditions. Microbiological testing and PCR assay testing for *Salmonella* followed the protocols described previously.

Iguanas in Group 1 were infected with 1.5×10^6 *Salmonella* Typhimurium strain 524, a nalidixic acid resistant mutant, in 1 ml of sterile saline *per os* fifteen days after the completion of the enrofloxacin treatment. The concentration of *Salmonella* organisms in the inoculum was estimated by streaking a sample on blood agar plates and counting colony forming units. Group 2 received an equal volume of 0.9% sterile saline per os. Cloacal-colon swabs were collected every seven days for 56 days and were placed into 7-ml of selenite enrichment broth and incubated at 37°C for 24 hours under aerobic conditions.

Iguanas were humanely euthanized fifty-seven days after completion of either *Salmonella* or saline treatment. The euthanasia, necropsy, and sample procedures were

the same as those previously described. Serotyping was performed on a limited number of *Salmonella* isolates to further characterize the organisms.

3.4.2 Sample Size Determination

Sample size was calculated under the following assumptions and criteria: that the proportion of *Salmonella*-positive iguanas in the infected treatment group was > 0.8, that the proportion of *Salmonella*-positive iguanas in the saline treatment group was < 0.3, that the $\alpha = 0.05$, and the power = 0.85 (Martin et al., 1987).

3.4.3 Statistical Analyses

The 95% binomial confidence intervals were calculated for the proportion estimates of the different treatment groups and sample specimens (cloacal swabs and necropsy specimens). In cases where the prevalence estimate was 0, the 95% confidence intervals were calculated with the technique described by van Belle and Millard (1998). The primary hypothesis evaluated in this study was that *Salmonella*-cleared green iguanas infected with *S.* Typhimurium strain 524 would be more likely to become *Salmonella*-positive than iguanas treated with saline (H_0: $\mu_I=\mu_S$; H_1: $\mu_I>\mu_S$). Comparison of the treatment groups (infection vs. saline) was made with the χ^2 test for homogeneity. The secondary hypothesis evaluated in this study was that *Salmonella* would be detected with a higher frequency from necropsy specimens than cloacal swabs (H_0: $\mu_C=\mu_N$; H_1: $\mu_C<\mu_N$). Comparison of the cloacal swabs and necropsy results for the *Salmonella* infected treatment group was made with the χ^2 test for homogeneity. Fisher's exact test was used to compare cloacal swabs and necropsy results for the saline control group because an expected cell value was < 5. Values of $p< 0.05$ were considered statistically different.

Statistical analysis was performed with EpiInfo 2000 (χ^2 test) (Centers for Disease Control, 2000) and StatExact 3 (Fisher's exact test) (Cytel Software, Cambridge, MA).

3.5 Effect of an Avirulent *Salmonella* Vaccine on the Colonization of *Salmonella* in Green Iguanas

3.5.1 Study Design and Sample Collection

In July 2000, an intervention study was performed at to determine if an attenuated *S.* Typhimurium could prevent colonization of *Salmonella* in young green iguanas. Iguanas were acquired from the commercial farm described in section 3.1.1. Two-hundred 3- to 4-month-old green iguanas collected by the farm employees with the techniques described previously were transported by air to Fluker Farms, Port Allen, LA, for *Salmonella* assay. Microbiologic culture and PCR assay techniques were similar to those described previously. An antimicrobial sensitivity profile for enrofloxacin and nalidixic acid was performed for each isolate as previously described.

One-hundred forty eight iguanas that were identified as *Salmonella*-positive, with an enrofloxacin and nalidixic acid sensitive isolate, were transferred from Fluker Farms to the LSU Life Sciences Vivarium. A physical examination was performed on each iguana and the animal's weight recorded. The iguanas were divided by random drawing into four groups: Group 1 (N=37)- negative control (not vaccinated and not infected with *Salmonella*), Group 2 (N=37)- vaccinated and not infected with *Salmonella*, Group 3 (N=37)- vaccinated and infected with *Salmonella*, and Group 4 (N=37)-positive control (not vaccinated and infected with *Salmonella*). The iguanas were maintained under the same conditions as described previously.

All 148 iguanas received enrofloxacin suspension (Baytril, Haver, Shawnee Mission, Kansas USA) (10 mg/kg PO SID) for 14 days. Cloacal swabs were collected every seven days for four weeks after enrofloxacin treatment, and were screened for *Salmonella* with microbiological and PCR assay techniques.

Iguanas in groups 2 and 3 were infected with 1.6×10^9 *S.* Typhimurium vaccine (Megan®Vac1, Megan Health, Inc., St. Louis, MO USA) in 1 ml of saline twenty-nine days after the completion of the enrofloxacin treatment. The concentration of *Salmonella* organisms in the inoculum was estimated by streaking a sample on blood agar plates and counting colony forming units. Iguanas in groups 1 and 4 received an equal volume of 0.9% sterile saline *per os*. Cloacal swabs were collected from all 148 iguanas every 7 days for 2 weeks post-vaccination. A booster vaccination (1.0×10^9 organisms/ml) was administered fourteen days after the initial vaccine to groups 2 and 3, while iguanas in groups 1 and 4 received an equal volume of 0.9% sterile saline *per os*. Cloacal swabs were again collected from all 148 animals for seven days after the booster vaccination period and screened for *Salmonella* with microbiological and molecular techniques as previously described.

One week after the booster vaccination, iguanas in groups 3 and 4 were infected with 1.5×10^8 organisms/ml of *S.* Typhimurium strain 524 in 1-ml of saline. The concentration of *Salmonella* organisms in the inoculum was estimated by streaking a sample on blood agar plates and counting colony forming units. Iguanas in groups 1 and 2 received 1 ml 0.9% sterile saline *per os*. Cloacal swabs were collected from all 148 animals every seven days for 21 days and screened for *Salmonella* with microbiological

and molecular techniques as previously described. All 148 iguanas were humanely euthanized 22 days after the *Salmonella*-challenge and samples collected from the lung, colon, small intestine, liver, spleen, and kidneys. The necropsy and sample processing were similar to the procedures described previously. All tissue samples were screened for *Salmonella* with microbiological and molecular techniques as previously described.

Fecal excretion of *S.* Typhimurium strain 524 from either cloacal swabs or necropsy specimens was used as an indicator of colonization and persistence in the gastrointestinal tract. Effectiveness of the vaccine was assessed by comparing the level of colonization of the viscera between unvaccinated and vaccinated iguanas. Immunological testing was not possible because of the limited size of the iguanas.

The attenuated vaccine strain of *S.* Typhimurium could be differentiated from other *Salmonella* on microbiological culture by colony morphology, a lack of H_2S production, and an inability to use citrate. The biochemical profile for the vaccine strain was similar to *Hafnia alvei*. The melting temperature for the probes used in the PCR assay for the vaccine strain of S. Typhimurium were similar to those observed for other salmonellae.

3.5.2 Sample Size Determination

Sample size was calculated under the following assumptions and criteria: That the proportion of *Salmonella*-positive iguanas in the unvaccinated and infected treatment group was > 0.8 after being infected, that the proportion of *Salmonella*-positive iguanas in the vaccinated and infected treatment group was <0.4 after being vaccinated and infected, that $\alpha = 0.05$, and that power = 0.92 (Martin et al., 1987).

3.5.3 Statistical Analyses

The 95% binomial confidence intervals were calculated for each of the proportion estimates of the different treatment groups and sample specimens. In cases where the prevalence estimate was 0, the 95% confidence intervals were calculated with the technique described by van Belle and Millard (1998). A χ^2 test for homogeneity was performed to evaluate the homogeneity of the treatment groups with respect to *Salmonella* status. The primary hypothesis evaluated in this study was that iguanas vaccinated with a commercial attenuated *Salmonella* vaccine and infected with *S.* Typhimurium strain 524 would be less likely to be *Salmonella*-positive than unvaccinated iguanas infected with the same strain of *Salmonella* (H_0: $\mu_V=\mu_{NV}$; H_1: $\mu_V<\mu_{NV}$). Comparison of the treatment groups (vaccinated vs. saline) was made with the χ^2 test for homogeneity. A secondary hypothesis was evaluated to determine if *Salmonella* would be detected at a higher frequency from necropsy specimens than cloacal swabs (H_0: $\mu_C=\mu_N$; H_1: $\mu_C<\mu_N$). Comparison of the cloacal swab and necropsy results for each treatment group was made with the χ^2 test for homogeneity. Fisher's exact test was used to compare cloacal swab and necropsy results for the saline control group because an expected cell value was < 5. Values of $p< 0.05$ were considered statistically different. Statistical analysis was performed with EpiInfo 2000 (χ^2 test) (Centers for Disease Control, 2000) and StatExact 3 (Fisher's exact test)(Cytel Software, Corp., Cambridge, MA).

CHAPTER 4

RESULTS

4.1 Epidemiologic Study of *Salmonella* in Green Iguanas on a Commercial Farm in El Salvador

Salmonella was isolated from 1,161 of 3,156 (Apparent prevalence (AP): 37%, 95% CI: 35-39; True prevalence (TP): 55%, 51-58%) samples derived from iguanas and their environment during the November and March collection periods (Tables 4-1 and Table 4-2).

In November 1999, *Salmonella* was isolated from 445 of 1,223 (AP: 36%, 95% CI: 33-39; TP: 53%, 48-58%) samples derived from iguanas and their environment. One-hundred fifty-one of 302 (AP: 50%, 95% CI: 44-56; TP: 74%, 65-83%) adult males and 129 of 405 (AP: 32%, 95% CI: 27-36; TP: 47%, 39-53%) adult female iguanas yielded a *Salmonella* on cloacal culture. Fifty-seven of 120 (AP: 47.5%, 95% CI: 39-56; TP: 70%, 57-83%) yearlings were *Salmonella*-positive. The gender of the yearlings could not be determined because they had not reached sexual maturity.

There was no evidence of *Salmonella* contamination of the feed, food preparation site, or food preparer's hands in the fall samples. *Salmonella* was isolated from 15 of 44 (AP: 34%, 95% CI: 20- 48; TP: 50%, 29-71%) water basins in twelve different pens (57%). Sixty-eight of 158 (AP: 43%, 95% CI: 35-51; TP: 64%, 51-76%) soil samples yielded *Salmonella*. *Salmonella* was isolated from the soil in 15 (71%) different pens. Pen MA-2 was the only pen from which *Salmonella* was not isolated from either soil or water. Insects found around the food preparation area did not yield

Salmonella, but the organism was isolated from the feces of all 18 wild-caught green iguanas and from 7 (AP: 78%, 95 %CI: 51-100; TP: 100%, 76-100%) amevia lizards.

Table 4-1
Summary of the *Salmonella* culture results from iguanas collected during two cross-sectional studies at a commercial iguanas farm in El Salvador.

Sample	Fall Sampling Period	Spring Sampling Period
Adult Male Iguanas	151/302	191/380
Adult Female Iguanas	129/405	183/407
Adult Female Necropsied	----------	20/25
Yearling Iguanas	57/120	
Male	---------	63/120
Female	---------	92/180
Hatchling Iguanas (1 month old)	---------	253/300
Hatchling Necropsied	---------	10/35
Embryo	---------	1/66
External egg surface	---------	16/66
Internal egg surface	---------	3/66

In March 2000 (Spring), *Salmonella* was isolated from 716 (AP: 37%, 95% CI: 35-39; TP: 55%, 51-58%) of 1,933 samples derived from iguanas and their environment. One-hundred ninety-one of 380 (AP: 50%, 95% CI: 45-55; TP: 74%, 67-82%) adult males yielded *Salmonella* on cloacal culture. One-hundred eighty-three of 407 (AP: 45%, 95% CI: 40- 50; TP: 67%, 59-74%) adult female iguanas captured from

two pens (IT-2 and IT-3) yielded *Salmonella* on cloacal culture. Ninety-four (AP: 45%, 95% CI: 38-52; TP: 67%, 56-77%) of 207 female iguanas from the IT-2 pen and 89 (AP: 44.5%, 95% CI: 38-51; TP: 66%, 56-76%) of 200 female iguanas from the IT-3 pen yielded *Salmonella* on cloacal culture. One-hundred fifty-five of 300 (AP: 52%, 95% CI: 46-58; TP: 77%, 68-86%) yearlings yielded *Salmonella* on cloacal culture. One-hundred eighty (60%) of the yearlings were female and 120 (40%) were male. Ninety-two (AP: 51%, 95% CI: 44-58; TP: 76%, 65-86%) of the female yearlings and 63 (AP: 52%, 95% CI: 43-61; TP: 77%, 64-91%) of the male yearlings yielded *Salmonella* on cloacal swab. Fifty (AP: 50%, 95% CI: 40- 60; TP: 74%, 59-89%) of the 100 yearlings from the MB-1 pen, 51 (AP: 51%, 95% CI: 41- 61; TP: 76%, 61-91%) of the 100 iguanas from the MC-1 pen, and 54 (AP: 54%, 95% CI: 44- 64; TP: 80%, 65-95%) of the 100 yearlings from the MD-3 pen yielded *Salmonella* on cloacal swabs.

There was no evidence of *Salmonella* contamination of the feed, food preparation site, or food preparer's hands in the spring samples. *Salmonella* was isolated from 28 of 72 (AP: 39%, 95% CI: 28-50; TP: 58%, 41-74%) water basins in 14 (67%) different pens. Eighty-one of 182 (AP: 44.5%, 95% CI: 37-52; TP: 66%, 54-77%) soil samples yielded *Salmonella* from the soil of fourteen different pens (67%). One (AP: 3%, 95% CI: 0-9; TP: 3%, 0-12%) of the 36 nest soil samples yielded *Salmonella*. Soil samples collected from the walls of the nest did not yield *Salmonella* (95% CI: 0-8). Five of 110 (AP: 5%, 95% CI: 0-9; TP: 6%, 0-12%) shelter structures yielded *Salmonella*. Insects found around the food preparation area did not yield *Salmonella*, but

Table 4-2

Summary of the *Salmonella* culture results from environmental samples collected during two cross-sectional studies at a commercial iguanas farm in El Salvador.

Sample	Fall Sampling Period	Spring Sampling Period
Rice	0/25	0/25
Wheat	0/25	0/25
Mixed feed samples	0/20	0/20
Mineral supplement	0/25	0/25
Vitamin, electrolyte supplement	0/20	0/20
Food preparer's hands	0/6	0/6
Grinders	0/18	0/18
Water samples		
well water	0/9	0/9
water basins	15/44	28/72
Surface Soil	68/158	81/182
Nest Soil	---------	1/36
Soil surrounding nest	---------	0/36
Shelters	---------	5/110
Flies	0/14	0/10
Wild lizards		
Iguana	18/18	2/3
Basilisk	0/1	1/1
Amevia	7/9	---------
Skink	0/1	0/3

the organism was isolated from the feces of two of three (AP: 67%, 95% CI: 14-100; TP: 100%, 20-100%) wild-caught green iguanas and 1 brown basilisk.

Twenty (AP: 80%, 95% CI: 64-96; TP: 100%, 95-100%) of the 25 adult female iguanas that were necropsied yielded *Salmonella* from at least one tissue (Table 4-3). Thirteen of the 25 small intestine samples (AP: 52%, 95% CI: 32-72; TP: 77%, 47-100%), 15 of 25 colon specimens (AP: 60%, 95% CI: 41-79; TP: 89%, 61-100%), 1 of 25 liver specimens (AP: 4%, 95% CI: 0-12; TP: 5%, 0-17%), and 1 of 25 ovary specimens (AP: 4%, 95% CI: 0-12; TP: 5%, 0-17%). *Chryseomonas luteola* and *Citrobacter braakii* were isolated from two different ovaries. There was no evidence of *Salmonella* in the oviduct of adult female iguanas (0/25; 95% CI: 0-12). *Proteus mirabilis* and *Citrobacter freundii* were isolated from two different oviducts.

Ten (AP: 29%, 95% CI: 14-44; TP: 42%, 20-65%) of the 35 hatchling iguanas yielded a *Salmonella* on at least one tissue (Table 4-4). Ten of 35 intestinal samples (AP: 29%, 95% CI: 14-44; TP: 42%, 20-65%), four of the 35 liver specimens (AP: 11%, 95% CI: 1-21; TP: 15%, 0-30%), and five of 35 internal yolk specimens (AP: 14%, 95% CI: 3-25; TP: 20%, 3-36%) from hatchling iguanas yielded a *Salmonella*.

Salmonella was isolated from the exterior of 16 of 66 egg shells (AP: 24%, 95% CI: 14- 34; 35%, 20-50%), and from at least one egg from 5 of the 8 clutches tested (62.5%). *Salmonella* was isolated from three of 66 (AP: 5%, 95% CI: 0-10; TP: 6%, 0-14%) embryonic yolk sacs prior to hatch. All three of these embryos also yielded *Salmonella* from the surface of the egg shell. *Salmonella* was isolated from one of 66

embryos (AP: 2%, 95% CI: 0-5; TP: 2%, 0-6%). The external egg surface and embryonic yolk sac of this subject were both *Salmonella*-negative.

Adult iguanas were less likely (OR: 0.77, 95% CI: 0.6-0.96) to be *Salmonella*-positive than yearling iguanas. Male iguanas were more likely (OR: 1.5, 95% CI: 1.2-1.8) to be *Salmonella*-positive than females. Cloacal swabs collected from the iguanas during the fall sampling period were less likely (OR: 0.7, 95% CI: 0.6-0.9) to be *Salmonella*-positive than the spring swabs. The stratum specific OR for adult males relative to yearling males was 0.9 (95% CI: 0.6-1.4) and 0.6 (95% CI: 0.4-0.85) for adult females relative to yearling females. There was no modification by gender of the effect of age class on *Salmonella* status (BD: 2.5, 1 df, p=0.1). The crude OR for age class (0.77; 95% CI: 0.6-0.96) and the MH weighted OR for age class (0.72, 95% CI: 0.6-0.9) were compared to evaluate gender as a confounder. The difference between the crude OR and the MH OR was 6.5%. Therefore, gender was not a confounder and the crude OR is reported (0.77; 95% CI: 0.6-0.96) . The stratum specific OR for adult iguanas relative to yearlings in the fall was 0.7 (95% CI: 0.5-1.1) and 0.8 (95% CI: 0.6-1.1) for adults relative to yearlings in the spring. Season did not modify the effect of age class on *Salmonella* status (BD: 0.45, 1 df, p=0.5). The crude OR for age class (0.77; 95% CI: 0.6-0.96) and the MH weighted OR (0.81, 95% CI: 0.6-1.0) were compared to evaluate season as a confounder. The difference between the crude OR and the MH OR was 5%. Therefore, season was not considered a confounder and the crude OR is reported (0.77; 95% CI: 0.6-0.96). Season did modify the effect of gender on *Salmonella* status (BD: 9.9, 1 df, p<0.001). The stratum specific OR for males relative to females in the fall was

2.1 (95% CI: 1.5-3.0) and for males relative to females in the spring was 1.1 (95% CI: 0.9-1.5). There was no significant difference in *Salmonella* recovery between water basin samples collected in the spring and fall (χ^2 : 0.27, 1 df, p=0.6) or from soil samples collected in the spring and fall (χ^2 : 0.07, 1 df, p=0.8).

4.2 Sensitivity and Specificity Estimation of Three Diagnostic Tests for *Salmonella* in Green Iguanas

Results of the study revealed two populations with apparently different prevalences of *Salmonella*. Applying the pre-shipment results, if microbiological culture had been used as the sole criteria to classify positive status, Farm 1 would have an estimated prevalence of 47.5% and Farm 2 would have an estimated prevalence of 22.5% (Table 4-5). Because PCR assay does not depend on viable organisms and the literature suggests that it is highly sensitive and specific, the results of the PCR assay were originally used to classify the positive status of an iguana. Based on the PCR assay, the estimated prevalences for Farm 1 was 97% and 33% for Farm 2 (Table 4-5). Applying the post-shipment results, if microbiological culture had been used as the sole criteria to classify positive status, Farm 1 would have an estimated prevalence of 53% and Farm 2 would have an estimated prevalence of 60% (Table 4-6). If ELISA had been the sole criteria to classify positive status, Farm 1 would have an estimated prevalence of 79% and Farm 2 would have an estimated prevalence of 73% (Table 4-7). Again, the PCR assay was used to classify positive status and the estimated prevalence for Farm 1 was 91% and 64% for Farm 2 (Table 4-6 and 4-7). The PCR assay estimates of the prevalence served as the prior information required for Bayesian analysis.

Table 4-3

Summary of the culture results from the adult female iguana necropsy specimens collected during the spring sampling period.

	Salmonella status				
Iguana Number	Ovary	Oviduct	Small Intestine	Colon	Liver
1	-	-	+	+	-
2	-	-	-	+	-
3	-	-	-	-	-
4	-	-	-	-	-
5	-	-	+	-	-
6	-	-	+	+	-
7	+	-	-	+	-
8	-	-	-	+	-
9	-	-	-	+	-
10	-	-	-	+	-
11	-	-	+	-	-
12	-	-	-	-	-
13	-	-	+	-	+
14	-	-	+	-	-
15	-	-	-	+	-
16	-	-	-	-	-
17	-	-	+	+	-
18	-	-	+	+	-
19	-	-	+	+	-
20	-	-	+	+	-
21	-	-	-	-	-
22	-	-	-	+	-
23	-	-	+	+	-
24	-	-	+	-	-
25	-	-	+	+	-

Table 4-4

Summary of the culture results from the hatchling iguana necropsy specimens from the spring sampling period.

	Salmonella status		
Iguana Number	Intestines	Liver	Yolk sac
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	+	+	-
6	-	-	-
7	-	-	-
8	-	-	-
9	-	-	-
10	-	-	-
11	-	-	-
12	-	-	-
13	-	-	-
14	-	-	-
15	-	-	-
16	-	-	-
17	-	-	-
18	-	-	-
19	-	-	-
20	+	-	-
21	+	-	-
22	-	-	-
23	+	+	+
24	+	+	+
25	+	+	+
26	+	-	-
27	-	-	-
28	-	-	-
29	+	-	+
30	+	-	+
31	-	-	-
32	-	-	-
33	-	-	-
34	-	-	-
35	+	-	-

Table 4-5

Cross-classification of pre-shipment PCR assay and microbiological culture results for *Salmonella*.

	Farm 1		Farm 2	
	Culture +	Culture -	Culture+	Culture -
PCR +	56	60	25	15
PCR -	1	3	2	78

Bayesian estimates were derived for each population independently. The results from the pre-shipment PCR and culture samples suggest that *Salmonella* infection between the two farms was different (Table 4-8). The estimated sensitivity and specificity for the PCR assay was consistent across the farms. The estimated specificity of culture was consistent across the two farms, but the sensitivity for microbiological culture was not. The estimated sensitivity for microbiological culture for Farm 1 was 0.64 with 95% credible intervals of 0.59-0.69, compared to 0.70 (0.65-0.74) for Farm 2. The 95% credible interval represents the range of values that contain the actual parameter mean. In this case, the 95% credible intervals for the two farm populations did not capture the median estimates for the populations, and more conservative estimates for the prior range of values representing the uncertainty in the culture sensitivity could have been used.

The estimated sensitivity and specificity for each assay was consistent for pre-shipment and post-shipment samples (Table 4-8, 4-9). Results of the prevalence and test parameter estimates derived from the simultaneous comparisons of the farms and the

PCR assay and microbiological culture, PCR assay and ELISA, and ELISA and microbiological culture, using both informative and non-informative priors are reported in Tables 4-10, 4-11, 4-12, and 4-13. Results with the informative priors were similar to the single farm comparisons. The estimated sensitivity of the microbiological culture for the pre-shipment samples was 0.65 (0.60-0.69) and was consistent with the single farm estimate for Farm 1. However, the estimated sensitivity of the microbiological culture for the post-shipment samples was 0.71 (0.67-0.75) and represented the two individual Farm estimates. When a non-informative prior estimate for prevalence was used, the parameter estimates for all the tests varied by 3-7 %

.

Table 4-6

Cross-classification of post-shipment PCR assay and microbiological culture results for *Salmonella.*

	Farm 1		Farm 2	
	Culture +	Culture -	Culture+	Culture -
PCR +	63	45	71	5
PCR -	0	11	1	41

Table 4-7

Cross-classification of post-shipment PCR assay and ELISA results for *Salmonella.*

	Farm 1		Farm 2	
	ELISA +	ELISA -	ELISA+	ELISA -
PCR +	90	18	71	5
PCR -	4	11	16	26

Table 4-8

Bayesian estimates (and 95% credible intervals) for pre-shipment PCR assay and microbiological culture results for *Salmonella* detection.

	Farm 1	Farm 2
Prevalence	0.99 (0.95-1.0)	0.33 (0.28-0.37)
PCR Sensitivity	0.93 (0.90-0.96)	0.90 (0.86-0.95)
PCR Specificity	0.95 (0.92-0.97)	0.95 (0.92-0.97)
Culture Sensitivity	0.64 (0.59-0.69)	0.70 (0.65-0.74)
Culture Specificity	0.99 (0.98-0.99)	0.99 (0.98-0.99)

Table 4-9

Bayesian estimates (and 95% credible intervals) for post-shipment PCR assay, ELISA, and microbiological culture results.

	Farm 1	Farm 2
Prevalence	0.92 (0.88-0.95)	0.66 (0.61-0.70)
PCR Sensitivity	0.93 (0.90-0.96)	0.92 (0.87-0.95)
PCR Specificity	0.94 (0.92-0.97)	0.95 (0.93-0.97)
ELISA Sensitivity	0.81 (0.77-0.85)	0.83 (0.79-0.87)
ELISA Specificity	0.90 (0.84-0.94)	0.86 (0.80-0.90)
Culture Sensitivity	0.67 (0.62-0.71)	0.74 (0.69-0.78)
Culture Specificity	0.99 (0.98-0.99)	0.99 (0.98-0.99)

To evaluate the possible effects of misclassification, data perturbations of the observed data were made and Bayesian estimation of the prevalence and test parameters repeated. The data perturbation results for the pre-shipment and post-shipment Bayesian estimates are shown in Tables 4-14, 4-15, 4-16, and 4-17. Data perturbation did not

affect the parameter estimates for sensitivity and specificity suggesting that the parameter estimates are robust

Table 4-10

Contrasting Bayesian estimates: two-population, two-test parameter estimates (and 95% credible intervals) and parameter estimates using non-informative prevalence priors (0.5) for pre-shipment samples.

	Bayesian estimation (Informative priors)	Bayesian estimation (Non-informative)
Prevalence Farm 1	0.99 (0.96-1.0)	0.98 (0.93-1.0)
Prevalence Farm 2	0.33 (0.28-0.37)	0.31 (0.23-0.41)
PCR Sensitivity	0.94 (0.90-0.96)	0.90 (0.86-0.93)
PCR Specificity	0.95 (0.93-0.97)	0.91 (0.86-0.95)
Culture Sensitivity	0.65 (0.60-0.69)	0.61 (0.57-0.66)
Culture Specificity	0.99 (0.98-0.99)	0.96 (0.93-0.97)

Table 4-11

Contrasting Bayesian Estimates: two-population, two-test parameter estimates (and 95% credible intervals) and parameter estimates using non-informative prevalence priors for post-shipment PCR assay and microbiological culture.

	Bayesian estimation (Informative priors)	Bayesian estimation (Non-informative)
Prevalence Farm 1	0.91 (0.87-0.95)	0.92 (0.85-0.97)
Prevalence Farm 2	0.65 (0.61-0.69)	0.67 (0.57-0.75)
PCR Sensitivity	0.95 (0.91-0.97)	0.91 (0.87-0.93)
PCR Specificity	0.95 (0.93-0.97)	0.92 (0.87-0.95)
Culture Sensitivity	0.71 (0.67-0.75)	0.67 (0.63-0.71)
Culture Specificity	0.99 (0.98-0.99)	0.96 (0.93-0.97)

Table 4-12

Contrasting Bayesian Estimates: two-population, two-test parameter estimates (and 95% credible intervals) and parameter estimates using non-informative prevalence priors for post-shipment PCR assay and ELISA.

	Bayesian estimation (Informative priors)	Bayesian estimation (Non-informative)
Prevalence Farm 1	0.93 (0.89-0.96)	0.94 (0.88-0.98)
Prevalence Farm 2	0.67 (0.62-0.71)	0.74 (0.64-0.82)
PCR Sensitivity	0.92 (0.88-0.95)	0.91 (0.87-0.94)
PCR Specificity	0.95 (0.93-0.97)	0.95 (0.93-0.97)
ELISA Sensitivity	0.83 (0.80-0.87)	0.83 (0.80-0.87)
ELISA Specificity	0.88 (0.82-0.93)	0.89 (0.83-0.93)

Table 4-13

Contrasting Bayesian Estimates: two-population, two-test parameter estimates (and 95% credible intervals) and parameter estimates using non-informative prevalence priors for post-shipment microbiological assay and ELISA.

	Bayesian estimation (Informative priors)	Bayesian estimation (Non-informative)
Prevalence Farm 1	0.89 (0.84-0.93)	0.85 (0.76-0.92)
Prevalence Farm 2	0.68 (0.63-0.72)	0.78 (0.69-0.86)
Culture Sensitivity	0.70 (0.66-0.74)	0.70 (0.66-0.74)
Culture Specificity	0.99 (0.98-0.99)	0.99 (0.99-0.99)
ELISA Sensitivity	0.85 (0.81-0.88)	0.85 (0.81-0.88)
ELISA Specificity	0.90 (0.85-0.94)	0.90 (0.85-0.94)

Table 4-14

Bayesian estimates for prevalence and test parameters (and 95% confidence intervals) from perturbed data from PCR assay and microbiological culture for pre-shipment samples.

	Observed values (56,60,1,3,25,15,2,78)	Perturbed data #1 (58,58,0,4,25,15,2,78)	Perturbed data #2 (55,58,4,3,24,13,5,78)
Prevalence Farm 1	0.99 (0.96-1.00)	0.98 (0.95-1.00)	0.99 (0.96-1.00)
Prevalence Farm 2	0.33 (0.28-0.37)	0.33 (0.28-0.37)	0.33 (0.28-0.37)
PCR Sensitivity	0.94 (0.90-0.96)	0.94 (0.90-0.96)	0.92 (0.88-0.96)
PCR Specificity	0.95 (0.93-0.97)	0.95 (0.92-0.97)	0.95 (0.93-0.97)
Culture Sensitivity	0.65 (0.60-0.69)	0.65 (0.61-0.69)	0.65 (0.61-0.69)
Culture Specificity	0.99 (0.98-0.99)	0.99 (0.98-0.99)	0.99 (0.98-0.99)

Table 4-15

Bayesian estimates for prevalence and test parameters (and 95% confidence intervals) from perturbed data from PCR assay and microbiological culture for post-shipment samples.

	Observed values (63,45,0,11,72,5,1,41)	Perturbed data #1 (65,43,2,9,72,5,1,41)	Perturbed data #2 (61,47,0,11,70,7,3,41)
Prevalence Farm 1	0.91 (0.87-0.95)	0.92 (0.88-0.95)	0.91 (0.87-0.95)
Prevalence Farm 2	0.65 (0.61-0.69)	0.65 (0.61-0.69)	0.65 (0.61-0.70)
PCR Sensitivity	0.95 (0.91-0.97)	0.94 (0.91-0.96)	0.94 (0.91-0.96)
PCR Specificity	0.95 (0.93-0.97)	0.95 (0.93-0.97)	0.95 (0.93-0.97)
Culture Sensitivity	0.71 (0.67-0.75)	0.71 (0.67-0.75)	0.70 (0.66-0.74)
Culture Specificity	0.99 (0.98-0.99)	0.99 (0.98-0.99)	0.99 (0.98-0.99)

Table 4-16

Bayesian estimates for prevalence and test parameters (and 95% confidence intervals) from perturbed data from PCR assay and ELISA for post-shipment samples.

	Observed values (90,18,4,7,71,5,16,26)	Perturbed data #1 (92,16,6,5,71,5,16,26)	Perturbed data #2 (89,20,3,7,73,4,15,26)
Prevalence Farm 1	0.93 (0.89-0.96)	0.93 (0.90-0.96)	0.93 (0.89-0.96)
Prevalence Farm 2	0.67 (0.62-0.71)	0.67 (0.62-0.71)	0.67 (0.62-0.71)
PCR Sensitivity	0.92 (0.88-0.95)	0.91 (0.87-0.94)	0.92 (0.89-0.95)
PCR Specificity	0.95 (0.93-0.97)	0.95 (0.92-0.97)	0.95 (0.93-0.97)
ELISA Sensitivity	0.83 (0.80-0.87)	0.84 (0.80-0.87)	0.83 (0.79-0.86)
ELISA Specificity	0.88 (0.82-0.93)	0.88 (0.82-0.93)	0.88 (0.82-0.92)

Table 4-17

Bayesian estimates for prevalence and test parameters (and 95% confidence intervals) from perturbed data from microbiological culture and ELISA for post-shipment samples.

	Observed values (57,37,6,19,70,18,3,28)	Perturbed data #1 (59,35,8,17,70,18,3,28)	Perturbed data #2 (56,39,5,19,72,17,2,28)
Prevalence Farm 1	0.89 (0.84-0.93)	0.90 (0.85-0.94)	0.89 (0.84-0.93)
Prevalence Farm 2	0.68 (0.63-0.72)	0.68 (0.63-0.72)	0.68 (0.63-0.72)
Culture Sensitivity	0.70 (0.66-0.74)	0.70 (0.66-0.74)	0.70 (0.66-0.74)
Culture Specificity	0.99 (0.98-0.99)	0.99 (0.98-0.99)	0.99 (0.98-0.99)
ELISA Sensitivity	0.85 (0.81-0.88)	0.84 (0.81-0.88)	0.85 (0.82-0.88)
ELISA Specificity	0.90 (0.85-0.94)	0.90 (0.84-0.94)	0.90 (0.85-0.94)

4.3 Establishing a *Salmonella* Clearance Model With Enrofloxacin

The results of the enrofloxacin clearance study are reported in Table 4-18. All 20 (95% CI: 0-15) of the iguanas treated with enrofloxacin were culture negative for *Salmonella* over three consecutive cloacal samples and at necropsy. One (5%, 95% CI: 0-15) cloacal sample from the enrofloxacin treatment group was PCR positive on the third cloacal swab post-treatment. All twenty of the cloacal samples from iguanas treated with enrofloxacin were PCR-negative at necropsy.

The entire study group (19/19) of iguanas receiving saline (positive controls) shed *Salmonella* at least once. Eleven (58%) of the positive control iguanas shed *Salmonella* as detected by cloacal swabs collected 1 day after the completion of the saline, 16 (84%) of the iguanas were positive on the second cloacal sample (7 days), and 14 (74%) of the iguanas were positive on the third cloacal sample (14 days). Seventeen (89%) of the positive control iguanas were culture-positive at necropsy. *Salmonella* was isolated from the small intestine (11/19), colon (11/19) and the liver (3/19) of the positive controls.

Seventeen (89%, 95% CI: 75-100) of the positive controls were *Salmonella* positive on the PCR assay at least once during the 14 day period after saline administration. Ten (53%) of the positive control iguanas yielded PCR-positive cloacal swabs collected 1 day after completing the saline, 13 (68%) of the iguanas were positive on the second cloacal sample (7 days), and 15 (79%) of the iguanas were positive on the third cloacal sample (14 days). Fifteen (79%) of the positive controls yielded PCR-

positive tissue samples at necropsy. *Salmonella* was detected in the small intestine (12/19), colon (12/19), and liver (7/19) of the positive controls.

Table 4-18
Summary of the results from the enrofloxacin study

Sample	*Salmonella*-positive Enrofloxacin Treatment Group (N=20)	*Salmonella*-positive Saline Treatment Group (N=19)
Cloacal swab		
Culture	0	19
PCR	1	17
Necropsy		
Culture	0	17
PCR	0	15

Iguanas treated with enrofloxacin were significantly less likely (x^2= 36.2, 1 df, p<0.0001) to be *Salmonella*- positive than iguanas treated with saline. There was no difference in the frequency of *Salmonella* recovery between cloacal swabs or necropsy specimens for either the enrofloxacin (Fisher's exact test: 0.96, 1 df, p=0.5) or saline treatment groups (Fisher's exact test: 1.7, 1 df, p=0.09).

4.4 Evaluation of the Infectivity of *Salmonella* Typhimurium Strain 524 in Iguanas Following Enrofloxacin Elimination of *Salmonella*

The results of the *Salmonella* infection model are reported in Table 4-19. None (0/19, 95% CI: 0-16) of the negative control iguanas (not infected with Salmonella) yielded *Salmonella* on microbiological culture over the experimental period of 70 days. One (5%, 95% CI: 0-15) cloacal sample from a negative control iguana was positive on PCR assay on the last cloacal swab. *Salmonella* was isolated on culture from 6 (32%,

95% CI: 11-53) of the negative control iguanas at necropsy. *Salmonella* was isolated from the small intestine (5/6), colon (3/6) and the blood (1/6) of the negative control iguanas. *Salmonella* was isolated from both the small intestine and colon in two of the iguanas. All of the *Salmonella* isolates were enrofloxacin and nalidixic acid sensitive. One (7%) of the negative control iguanas yielded a positive PCR assay from a colon sample at necropsy. Five of the PCR necropsy samples could not be evaluated because the samples were mislaid during transport to the University of Georgia. The original *Salmonella* isolate and the isolate obtained at necropsy from each of the control iguanas that were *Salmonella* positive at the end of the study were submitted to the National Veterinary Services Laboratories in Ames, IA to be serotyped. In all six cases, the serotype isolated at the end of the study was different from the original isolate (Table 4-20).

Table 4-19

Summary of the results from the infection study

Sample	*Salmonella*-positive *Salmonella*-Infected Treatment Group (N=19)	*Salmonella*-positive Saline Treatment Group (N=19)
Cloacal swab		
Culture	9	0
PCR	10	1
Necropsy		
Culture	17	6
PCR	17	6*

* N=14

Table 4-20

Salmonella serotypes isolated from the negative control iguanas at the beginning and termination of the infection study

Iguana number	Preliminary serotype	Necropsy serotype
40	*S.* 50: B76	*S.* Typhimurium
43	*S.* Hagenbeck	*S.* Chameleon
44	*S.* L, V-Z35 (Arizona)	*S.* G, Z51
72	*S.* 50: B-Z6	*S.* Chameleon
78	*S.* 58: L, V-Z35 (Arizona)	*S.* Chameleon
90	*S.* 50: B-Z6	*S.* Chameleon

Nine (47%, 95% CI: 24-69) of the 19 infected iguanas shed *S.* Typhimurium strain 524 at least once after being infected with a culture of this organism. *Salmonella* Typhimurium strain 524 was isolated from seventeen (90%, 95% CI: 76-100) of the infected iguanas at necropsy with isolates recovered from the small intestine (16/17), colon (14/17) and liver (1/17). *Salmonella* was isolated from both the small intestine and colon in 13 of the infected iguanas. Ten (52%; 95% CI: 29-74) of the infected iguanas were PCR positive for *Salmonella* at least once during the study and seven (37%, 95% CI: 15-59) of the infected iguanas were PCR positive for *Salmonella* at necropsy. The positive samples were identified from the following tissues: colon (4/7), spleen (2/7), small intestine (1/7) and blood (1/7).

Iguanas infected with *S.* Typhimurium strain 524 were significantly more likely (χ^2= 13.3, 1 df, $p<0.001$) to be *Salmonella* positive than iguanas treated with saline.

Necropsy specimens from both the *Salmonella* infection group (χ^2= 6.3, 1 df, p<0.01) and the saline treatment group (Fisher's exact test: 4.2, 1 df, p<0.05) were significantly more likely to yield a positive result than cloacal swabs.

4. 5 Effect of an Avirulent Salmonella Vaccine on the Colonization of Salmonella in Green Iguanas

All 148 (100%) iguanas were culture negative for 28 days after the enrofloxacin treatment (Table 4-21). One (3%; 95% CI:0-8) of the Group 1 iguanas (negative control) and two (5%, 95% CI: 0-12) of the Group 2 iguanas (vaccine negative controls) yielded a *Salmonella* on cloacal swab during the 70-day experimental period. *Salmonella* was isolated from the small intestine or colon from three (8%, CI: 0-17) of the Group 1 iguanas and two (5%, CI: 0-12) of the Group 2 iguanas at necropsy and the isolates were sensitive to both enrofloxacin and nalidixic acid. The two positive PCR assays from cloacal swabs and the one PCR positive necropsy specimen from the Group 2 iguanas may have represented the vaccine strain, as microbiological culture was negative. Four (11%, 95% CI: 1-21) different iguanas from group 1 and six (16%, 95% CI: 4-28) different iguanas from Group 2 yielded *Salmonella* from either a cloacal swab or necropsy specimen. Seventeen (46%, 95% CI: 30-62) of the 37 Group 3 iguanas (vaccinated and infected) yielded *S.* Typhimurium strain 524 at least once after being infected. *Salmonella* Typhimurium strain 524 was isolated from 11 (30%, 95% CI: 15-45) of these iguanas at necropsy. Twenty-one (57%, 95% CI: 41-73) different iguanas from Group 3 yielded *S.* Typhimurium strain 524 from either cloacal swabs or necropsy specimens.

Table 4-21

Summary of the results from the vaccine study.

Sample	*Salmonella*-positive			
	Group 1 (N=37)	Group 2 (N=37)	Group 3 (N=37)	Group 4 (N=37)
Cloacal swab				
Culture	1	1	17*	13*
PCR	1	2	12	5
Necropsy				
Culture	3	2	11*	13*
PCR	0	1	4	5
No. Individual Iguanas	4	6	21	21

Group 1: no vaccine, saline
Group 2: vaccine, saline
Group 3: vaccine, infected *S.* Typhimurium
Group 4: no vaccine, infected *S.* Typhimurium
* *Salmonella* Typhimurium strain 524

Thirteen (35%, 95% CI: 20-50) of the 37 Group 4 iguanas (un-vaccinated and infected iguanas) shed *S.* Typhimurium strain 524 at least once after being infected. *Salmonella* Typhimurium strain 524 was isolated from 13 (35%, 95% CI: 20-50) iguanas at necropsy. Twenty-one (57%, 95% CI: 41-73) different iguanas from Group 4 yielded *S.* Typhimurium strain 524 from either cloacal swabs or necropsy specimens. The vaccine strain of *Salmonella* was isolated from the lungs of four (5%) of the vaccinated iguanas at necropsy. Three of the isolates were from the Group 2 (vaccine controls) and one of the isolates was from Group 3 (vaccinated and infected group).

At least two of the treatment groups were not homogeneous with respect to *Salmonella* status (χ^2= 31.2, 3 df, $p<0.0001$). There was no difference in the *S.* Typhimurium strain 524 status between Group 3 (vaccinated) and Group 4

(unvaccinated) iguanas (χ^2= 0.06, 1 df, p=0.8). There was no difference in *Salmonella* detection between cloacal swabs and necropsy specimens for group 1 (Fisher's exact test: 0.99, 1 df, p=0.16), group 2 (Fisher's exact test: 0.08, 1 df, p=0.38), group 3 (χ^2= 2.1, 1 df, p=0.07) or group 4 (χ^2= 0.00, 1 df, p=0.5).

CHAPTER 5

DISCUSSION

5.1 Epidemiologic Study of *Salmonella* in Green Iguanas on Commercial Farm in El Salvador

The overall objective of this study was to describe the epidemiology of *Salmonella* in commercially bred green iguanas that are exported to the USA. A specific aspect relating to the epidemiology of the infection concerned the potential for vertical and horizontal transmission.

The presence of *Salmonella* in the hatchling, yearling, and adult iguanas is consistent with previous reports of isolates from both cloacal and fecal specimens (CDC, 1992b). The prevalence of *Salmonella* in the adult population of iguanas (44%) conforms to the results of other studies (Cambre, 1980; Onderka and Finlayson, 1985). The prevalence reported in this population may be an underestimate of the true prevalence, as it is based on single cloacal samples. Shedding of *Salmonella* is transient in reptiles and repeated assays are required to establish the presence of infection (Burnham et al., 1998).

Adult iguanas (44%) were less likely to be *Salmonella* positive on cloacal swab than yearlings (50%). The difference in prevalence may be due to stocking density and stress. Up to 6,000 yearling iguanas were housed in small pens on the farm (9-10 iguanas/ m^2) in comparison to adult iguanas which were housed in large pens (2-3 iguanas/m^2). Farm employees enter the yearling iguana pens regularly to capture animals for export. The stress associated of high density, in combination with repeated exposure

to humans, may explain the increased shedding of *Salmonella* documented in yearlings. A large amount of fecal material was present in yearling pens during collection of the soil samples. The high stocking density, coupled with exposure to feces, leads to continuous exposure of yearlings to *Salmonella* for yearlings. Although there was a difference in the prevalence between the two age classes, the biological importance of a 6% difference is unknown.

The recovery of *Salmonella* from the 1- to 3-day old hatchlings at necropsy, and the cloacal swabs collected from the 1-month old iguanas, are significant. Reptiles absorb internalized yolk as a source of nutrients and probably do not consume food within the first week after hatching. The iguana tongue is a chemosensory organ and oral exposure to *Salmonella* could occur from contact with infected soil in nests or pens while investigating their environment. Recovery of *Salmonella* in 29% (10/35) of the intestinal tracts of 1- to 3-day old hatchlings suggests that these animals are exposed to *Salmonella* in their environment during the immediate post-hatch period. The green iguana is both geophagic and coprophagic and hatchlings acquire intestinal microflora from droppings of mature iguanas (Sokol, 1971). Under natural conditions, iguana hatchlings would consume fecal material from adults older than 90 days. Unless weather conditions were extreme, *Salmonella* would be expected to survive over this period of time (Morse and Duncan, 1974). Feces are not removed from breeding pens after females have deposited their eggs. The intestinal microflora of naive hatchling iguanas probably comprises opportunistic organisms encountered in the environment. Chicks and immature mice are more susceptible to colonization of environmental opportunistic

pathogens than adult animals with an established microflora (Sadler et al., 1969; Lee et al., 1972). The high prevalence (84%) of *Salmonella* reported in the 1-month old hatchlings, in combination with frequent isolation of *Salmonella* from soil and water, supports the theory that horizontal transmission is an important source of *Salmonella* for these reptiles. Regular removal and disposal of fecal material should be evaluated as a means to reduce exposure.

Male iguanas (50%) were more likely to be *Salmonella* positive than female iguanas (38%) and season modified the effect of gender on *Salmonella* status. Male and female iguanas are housed separately throughout the year, except during the breeding season (October- January). The overall prevalence of *Salmonella* in adult males (50%) was higher than adult females (32%) in the fall. Folliculogenesis commences between October and November (Rand, 1982), and the ovaries increase markedly in size during folliculogenesis and eventually distend the coelomic cavity. During this time, females fast and the lower prevalence of *Salmonella* shedding observed in female iguanas may be associated with fasting, which alters the balance and composition of intestinal flora. During the spring season, after females have deposited their eggs and resumed eating, the difference in prevalence is not as evident (Male: 50%, Female: 45%).

Recovery of *Salmonella* from 80% (20/25) of adult female iguanas at necropsy suggest that the prevalence of *Salmonella* in adult females reported from the single-cloacal swab may be an underestimate of true prevalence. Recovery of *Salmonella* from the ovary of one female, 14% (5/35) from the internalized yolk-sacs of the hatchlings, 5% (3/66) from the embryonic yolk sacs, and one embryo suggest that this organism

may be transmitted vertically. In other captive reptiles, including red-ear slider turtles (*Trachemys scripta elegans*) and Northern water snakes (*Natrix sipedon sipedon*), *Salmonella* has been isolated from the oviduct, the interior of the egg and the embryo (Kaufman and Morrison, 1966; Chiodini, 1982). Recovery of *Chryseomonas luteola* and *Citrobacter braakii* from ovaries of two different iguanas suggests that bacterial contamination of the gonad is not a rare event.

The relatively high presence of *Salmonella* detected on the external surface of eggs (34%), in contrast to the low number of isolates from the yolk sac (5%), suggests that eggs are contaminated in passing through the proctodeum, which is in close proximity to the coprodeum. Surface contamination of the shell is a mechanical process dependent on colonization of the terminal intestinal tract. Five of the eight clutches (62.5%) examined had at least one egg that yielded *Salmonella*. It is feasible that one egg could serve to infect an entire clutch as the hatchlings lick and eat the egg shell remnants (Troyer, 1982).

The absence of *Salmonella* contamination of feed in both the spring and fall sampling periods suggests that diet was not a source of infection in this commercial operation on those sampling days. The potential for food to serve as a source of *Salmonella* contamination should always be considered. The commercial grain used to feed iguanas, including rice and wheat, are processed at a local grain mill. These feed items can become contaminated by other ingredients if strict sanitary procedures are not followed during manufacture. Vegetables can be contaminated with *Salmonella* through growing or harvesting, or subsequently during post-harvest processing and distribution

(Beuchat and Ryu, 1996). In El Salvador, vermin, insects, and birds were routinely observed in the planted fields surrounding the iguana farm. If attempts are made to establish *Salmonella*-free iguanas, regular assay of food ingredients intended for the unit should be a routine quality control procedure.

Recovery of *Salmonella* in the water basins during the fall (34%) and spring (41%) suggests that these receptacles may serve as a source of infection. Iguanas were observed at the water basins regularly throughout the day. Iguanas were observed soaking in the water basins which contained feces. It has been suggested that this behavior stimulates gastrointestinal motility resulting in defecation. Restricting access to the water basins by providing closed watering systems as used for poultry, swine, and calves and by constructing physical barriers to inhibit entry to open basins could reduce the horizontal transmission of *Salmonella* in this commercial operation.

The high prevalence of *Salmonella* in wild-caught green iguanas (95%) and amevias (78%) captured on the farm suggests that these species serve as potential reservoirs of infection for captive breeding iguanas and their progeny. Although captured outside the pens, employees also reported observing these lizards within the pens. Mature wild-caught iguanas are routinely captured by employees and eaten. Workers that handle these reptiles may serve as a potential source of *Salmonella* exposure for captive iguanas because their personal hygiene practices are questionable. Other potential sources of environmental contamination include free-ranging birds and rodents, although none of these animals were trapped. Rats and mice were not considered a problem on the farm since initiating control methods in 1996.

Although serotyping of *Salmonella* was not performed in this investigation, a pilot study performed in March 1998 at the same commercial operation revealed eight different serotypes from 19 different samples (Mitchell and Shane, 2000). *Salmonella* serotyping is used to identify the potential point-source of an infection (CDC, 1995). At this unit, the multiple serotypes isolated from both iguanas and their environment make it difficult to identify a single point source that would form the basis for control methods. In the 1998 study, single colonies were selected for serotyping. Burnham et al. (1998) isolated more than one serotype in 24% (21/88) of the fecal samples from iguanas they examined, suggesting that the number of serotypes isolated at the commercial farm are underestimated.

Because of the high carriage rate of *Salmonella* in green iguanas, prospective owners should be informed of the potential risks associated with zoonotic infection. Washing of hands after handling iguanas is strongly recommended (Cambre and McGuill, 2000). Providing an appropriate environment and adequate nutrition for the pet iguana is also important to maintain health. Iguanas are inappropriate pets for immunocompromised owners and in households with young children (Cambre and McGuill, 2000).

5.2 Sensitivity and Specificity Estimation of Three Diagnostic Tests for *Salmonella* in Green Iguanas

A number of diagnostic assays are used to characterize the *Salmonella* carrier status of domestic animals. These include PCR, ELISA, and culture assays (Cohen et al., 1994b, Isaacson et al, 1999; Pelton et al, 1994). Published reports relating to the

Salmonella status of reptiles have been limited to microbiologic culture and ELISA (Burnham et al., 1998; Cambre et al., 1980, Pelton et al., 1994; Onderka and Finlayson, 1985). There have been no formal studies to estimate the test characteristics for the various diagnostic assays for *Salmonella*. The objectives of this study were:

- to estimate the prevalence of *Salmonella* in two populations of hatchling iguanas scheduled for export to the United States
- to estimate the test sensitivity and specificity for a polymerase chain reaction assay, an enzyme-linked immunosorbent assay, and microbiologic culture with the Bayesian estimation technique

The PCR assay was more likely to classify a sample as *Salmonella*-positive than either the ELISA or microbiologic culture. The PCR assay detects DNA, ELISA demonstrates antigen, and culture identifies live organisms. The number of organisms required to detect *Salmonella* with a PCR assay may be as low as one organism per gram of feces (Cohen et al. 1994b), although the detection level for the PCR assay used in this study was 10^4 organisms/ml. The detection level for the commercial ELISA was 10^5 to 10^6 organisms/ml. Differences detected between the PCR assay and ELISA may be attributed to the detection sensitivity of the test. The minimum detection level for culture was 10^3 to 10^4 organisms/ml. The similarity in the detection levels between the PCR and culture assays suggest that the difference observed between the results was not associated with the sensitivity of the tests. Because pre-shipment cloacal samples were transported to the United States for analysis, it is feasible that *Salmonella* in some samples did not survive transport. The PCR assay does not distinguish between live and

dead organism, whereas live organisms are necessary for culture. Test agreement between PCR and microbiologic culture was 49% for pre-shipment samples and 95% for post-shipment samples, suggesting that the status of the *Salmonella* may have affected the outcome of the testing procedure. The ELISA procedure was more likely to classify a sample as *Salmonella*-positive than culture. The ELISA, as with the PCR assay, does not require viable organisms to confirm diagnosis. The higher estimated ELISA sensitivity may be attributed to detection of non-viable organisms.

Results of this study clearly show that infected iguanas may be misclassified as false negatives by microbiological culture. According to sensitivity estimates for microbiological culture derived with the Bayesian methods, as many as three to four *Salmonella*-positive iguanas out of ten could be misclassified as *Salmonella*-negative if microbiological culture was used as a sole screening test. This is an important finding because most of the published reports characterizing *Salmonella*-status of reptiles are based on microbiologic culture, and results may be an underestimate of the true prevalence. The high estimated sensitivity and specificity of the PCR suggest that it would be the preferred assay among the three procedures if only one test was available.

The difference in the prevalence rates between the two populations of iguanas requires comment. Both farms are located in a similar geographical area in El Salvador, adjacent to the Pacific coast. The iguanas are housed in similar pens and are fed similar diets with common ingredients. The primary differences between the two iguana farms relate to the breeding stock, the water supply obtained from a well on each farm, and the employees. The field epidemiologic study discussed in section 3.1 was conducted at

Farm 1. Samples of the well water, and culture swabs from the surface of the food preparers' hands, were negative for *Salmonella* on microbiological culture. A cross-sectional study to evaluate recovery of *Salmonella* from the iguanas and their environment at Farm 2 would be useful to further evaluate the differences in population prevalence observed in this study.

Prevalence of *Salmonella* in iguanas from Farm 2 doubled between the time of pre-shipment collection (33%) and post-shipment collection (66%). No difference was observed in the Farm 1 population, however the pre-shipment prevalence was very high (97%). Burnham et al. (1998) reported that iguanas may shed *Salmonella* in their feces continuously or intermittently, however there have been no reports in the literature addressing the effect of transport on shedding of *Salmonella* in green iguanas. Williams and Newell (1970) reported that naturally-infected pigs were more likely to shed *Salmonella* after being transported and Isaacson et al. (1999) reported similar findings in experimentally infected animals. The increased frequency in shedding detected in post-shipment samples from iguanas in this study suggests that transport may increase the risk of exposure to *Salmonella* for prospective caretakers and pet owners. Unfortunately, because the purpose of this study was to evaluate diagnostic tests, rather than the effect of transport on the shedding, a control group was not evaluated. Future research to evaluate the effect of stress events, such as transportation, feed withdrawal, and hypothermia, on *Salmonella* shedding should be pursued.

The PCR assay clearly showed the most sensitivity as a diagnostic test for *Salmonella*. The estimated test sensitivity and specificity for PCR suggest that

misclassification should be reduced with this assay. The two limits associated with the PCR assay are an inability to compare serotypes and availability of the procedure. The PCR assay cannot be used to serotype *Salmonella*. Public health investigators screening iguanas for a specific *Salmonella* serotype in a reptile-associated zoonotic infection must still rely on microbiologic culture to confirm a source of infection. Clinical microbiologic laboratories routinely perform ELISA and culture assays for *Salmonella*. Generally, PCR assays are only offered by research and contract laboratories. Different primers used for the PCR assay may result in interlaboratory differences in results. Parallel testing using both microbiologic culture and the PCR assays will increase the sensitivity of the testing procedure and also yield isolates for subsequent serotyping.

5.3 Establishing a *Salmonella* Clearance Model With Enrofloxacin

The propagation of *Salmonella*-free iguanas is a primary objective for iguana farmers and is a concern of public health officials. Because of the widespread contamination of *Salmonella* on the iguana farm in El Salvador, and the necessity to maintain and multiply iguanas in outdoor pens, elimination of *Salmonella* at the farm would be difficult, if not impossible. Another approach to eliminating the risk of iguana-associated salmonellosis for pet owners is to reduce infection rates in the hatchling iguanas. Because young iguanas serve as sources of infection for pet owners, neonates are a logical starting point for programs focused on suppressing or eliminating *Salmonella*. Objectives of this study were to evaluate enrofloxacin as a method to suppress or eliminate *Salmonella* in immature green iguanas in a controlled

environment and to develop a *Salmonella* clearance model to evaluate possible methods of control.

Administration of enrofloxacin suspension at 10 mg/kg *per os* for 14 days effectively eliminated *Salmonella* from the treated iguanas. The results are consistent with those reported for poultry and calves (Bauditz, 1987). This finding is significant because reports in the literature suggest that antibiotic therapy promotes development of resistant strains of *Salmonella* or suppresses the organisms temporarily, with subsequent shedding (Cambre and McGuill, 2000). Veterinarians use antimicrobials to treat a variety of non-*Salmonella* bacterial infections in reptiles. In theory, if an antimicrobial is selected on the basis of its antimicrobial sensitivity profile, and the drug is administered for an appropriate length of time, and if necessary hygiene and environmental control methods are maintained, then eradication of the target pathogen should be possible. All of the *Salmonella* isolates obtained from iguanas enrolled in this study were susceptible to enrofloxacin. Iguanas enrolled in this study were maintained in a *Salmonella*-free environment and were fed a commercial iguana feed shown to be *Salmonella*-free and received chlorinated tap water. It is not known what the re-infection rate would have been if the subjects would have been re-exposed to *Salmonella.*

The positive PCR assay on the cloacal swab of the iguana from the enrofloxacin-treatment group is an interesting finding. This iguana was negative on PCR assay for *Salmonella* on the first two cloacal swabs and at necropsy. The PCR assay was repeated and the sample was again positive. Based on the findings of the Bayesian analysis (section 4.2), the estimated specificity of the PCR assay

approximately 95%. The high specificity of the PCR assay suggests that a false positive test result is unlikely. The PCR assay is reported to be capable of detecting as few as one organism per gram of feces (Cohen et al., 1993; Cohen et al., 1994a), which is below the threshold of detection for culture. The sensitivity of the PCR assay used for this study was 10^4 CFU/ml, similar to culture. Because selenite enrichment broth was used, the numbers of organisms would have been increased to levels greater than 10^4 colony forming units/ml. The positive cloacal swab assay could have represented dead salmonellae excreted from the iguana, as the assay detects DNA from both living or dead organisms. Reverse transcriptase PCR (RT-PCR) assay detects messenger RNA, hence a live organism. Unfortunately, RT-PCR analysis was not available for detection of *Salmonella* in this study. Gastrointestinal motility of herbivorous lizards is slower than mammals, and delayed emptying of the gastrointestinal tract may explain the positive PCR assay 14 days after completion of the enrofloxacin treatment.

All of the positive control iguanas yielded *Salmonella* during at least one sample period. There was no difference in recovery of *Salmonella* from cloacal swabs or necropsy specimens. Two iguanas which shed *Salmonella* during the study, did not yield *Salmonella* at necropsy. The necropsy samples included bile and feces, which were presumably contaminated with intestinal flora that could compete with *Salmonella* for the micronutrients in the enrichment broth. The findings from the cloacal swabs confirm that iguanas shed *Salmonella* periodically and this reinforces the importance of sequential samples to characterize *Salmonella* status of an iguana.

Culture and PCR assays were used simultaneously to increase the sensitivity of the testing protocol. The procedures were performed on both samples collected from the enrichment broth. The culture assay was performed immediately, while the PCR assays were conducted on samples subjected to refrigerated storage until shipment to the University of Georgia at the end of the study. The difference in time in performing the PCR assay may have affected the sensitivity of the assay. Fecal material (swab) and tissue contain inhibitors that may affect the PCR assay. Degradation of the DNA of *Salmonella* might be expected to occur over time.

5.4 Evaluation of the Infectivity of *Salmonella* Typhimurium Strain 524 in Iguanas Following Enrofloxacin Elimination of *Salmonella*

Based on the epidemiologic studies conducted at the iguana in El Salvador, (section 5.2), it is concluded that the majority of captive iguanas are exposed to *Salmonella* through horizontal transmission. There have been no formal studies to confirm the infectivity of *Salmonella* via the oral route in the iguana. The objectives of this specific study were to determine if immature iguanas could be infected using an oral inoculum of *Salmonella*, to create an infection model that could be used to evaluate methods to eradicate *Salmonella*, and ascertain the long term effects of administering enrofloxacin on eliminating *Salmonella* colonization of the intestinal tract in the iguana.

The results of this study confirmed that iguanas can be infected with an oral inoculum of *Salmonella*. The distribution of *Salmonella* in infected iguanas included the small intestine, colon, spleen, liver and blood. Colonization of the gastrointestinal tract was not tissue specific, as the colon and small intestine were colonized in thirteen

of the infected iguanas, consistent with the surveys conducted on snakes (Chiodini, 1982). All of the cloacal swab and necropsy isolates had an antibiotic sensitivity profile consistent with *S.* Typhimurium Strain 524, the infective organism.

There were no obvious clinical changes observed in the iguanas infected with *S.* Typhimurium strain 524. This is consistent with the fact that there were no obvious gross lesions observed at necropsy in any of the infected iguanas. Chiodini (1982) reported that snakes infected with an oral inoculum of *Salmonella* excreted the organisms in their feces with no adverse effects of infection. The snakes in the experiment did not develop an antibody response after receiving an oral inoculum, but developed an antibody response when *Salmonella* was injected into the coelomic cavity. Bichler et al. (1996) infected White Leghorn chickens with *Salmonella* without any adverse clinical response in the recipients. *Salmonella* infection in poultry is widely recognized and is a function of housing system and the composition of the gastrointestinal microflora. Chicks that are infected with *Salmonella* at 1-day of age are more susceptible to infection than older chickens (Sadler et al., 1969), consistent with the fact that neonates and animals with a naive or compromised gastrointestinal flora are more susceptible to *Salmonella* colonization through absence of competitive exclusion. In this study, enrofloxacin likely altered or markedly suppressed the intestinal microflora of the iguanas and provided the opportunity for colonization with *Salmonella.*

The importance of collecting sequential cloacal swab samples from iguanas to establish the *Salmonella* colonization status is confirmed by the pattern of excretion of

experimentally infected subjects. *Salmonella* Typhimurium strain 524 was isolated from two (10%) infected iguanas 7 days after being challenged. Three weeks after infection, an additional 2 iguanas were shedding *S.* Typhimurium strain 524. By the fourth week post-infection, nine iguanas (45%) were shedding *S.* Typhimurium strain 524.

This study illustrates the limits of using antimicrobials to control *Salmonella*. Seventeen iguanas treated with enrofloxacin at the beginning of the study were successfully infected with *S.* Typhimurium strain 524. The fact that these iguanas were re-infected suggests that there was no protective immunity after infection. Future research to assess the immune response of iguanas should be pursued.

None of the non-infected control iguanas treated with enrofloxacin yielded a *Salmonella* from a cloacal sample during the experiment, but six of these iguanas were positive at necropsy. This aspect of the study confirms that iguanas treated with antibiotics can develop a latent infection. In this study, *Salmonella* shedding was suppressed for at least 70 days in infected iguanas. All six isolates obtained at necropsy were a different serotype compared to the original isolates obtained at the time of enrollment into the study (Table 4-20). These six isolates were sensitive to enrofloxacin. This is an important observation because it confirms that treatment is not necessarily associated with the development of antimicrobial resistance. Because *Salmonella* is a facultative intracellular pathogen, some organisms may have been refractory to enrofloxacin. It is possible that these isolates were present on the media at the time of isolation, but were not selected for subsequent characterization and classification. Sequential samples should be collected to characterize the actual *Salmonella* status of an

iguana. Burnham et al. (1998) reported that a minimum of three fecal swabs should be collected at one week intervals to assess *Salmonella* status. The finding in this study indicate that treatment with an antibiotic prior to testing may suppress shedding up to ten weeks post-treatment. Public health investigators screening iguanas for a specific *Salmonella* serotype in a reptile-associated zoonotic infection should collect multiple colonies for serotyping to improve the probability of identifying the source of the infection.

The positive PCR assay on the cloacal swab from the iguana derived from the negative control group requires explanation and comment. This iguana was negative on the PCR assay for *Salmonella* over the first five sequential cloacal samples, but was positive on the last swab. Unlike the negative control iguana in the enrofloxacin study that tested PCR positive, *Salmonella* was isolated from the small intestine of this iguana at necropsy. Unfortunately, the PCR assays of the specimens obtained at necropsy were not performed because the samples were unavailable. A positive PCR assay may have predicted onset of shedding.

Salmonella was isolated more frequently from the necropsy samples than the cloacal samples. This would be expected because intestinal shedding is a transient occurrence. Entire organs or tissue samples collected at necropsy contain *Salmonella* organisms which have an intracellular distribution, and would be more likely to be detected following maceration and enrichment.

5.5 Effect of an Avirulent *Salmonella* Vaccine on the Colonization of Salmonella in Green Iguanas

Salmonella colonization of the intestinal tract of young iguanas derived from the farm in El Salvador is evidently widespread. Because *Salmonella* is endemic on the farm, it can be readily transmitted among the iguanas and other reservoirs. The administration of antimicrobials to iguanas in a *Salmonella*-free, controlled environment had variable results. Although *Salmonella* colonization was eliminated from the majority of the iguanas treated with enrofloxacin, a small proportion of the population yielded *Salmonella* at necropsy. Another shortcoming of using antimicrobials to eradicate *Salmonella* is that they do not provide any long-term protection, as was observed in the infection study. Vaccination programs to protect breeding animals and their progeny are applied inconsistently in the poultry industry to suppress *Salmonella.* The objective of this study was to evaluate a live attenuated mutant *Salmonella* vaccine as a method to suppress or eliminate *Salmonella* infection in immature green iguanas.

Three (8%) of the iguanas from Group 1 and two (5%) of the iguanas from Group 2 were *Salmonella*-positive at necropsy. This finding is consistent with the results from the infection study, that iguanas treated with antibiotics can develop a latent infection. All of the isolates obtained at necropsy were sensitive to enrofloxacin and nalidixic acid. This is an important observation because it confirms the findings that treatment is unrelated to the development of resistance to colonization. Again, this study showed that treatment with an antibiotic may suppress shedding for up to ten weeks post-treatment.

There was no difference in the recovery of *S.* Typhimurium strain 524 between vaccinated and infected iguanas (Group 3) and unvaccinated and infected iguanas (Group 4). The commercial attenuated vaccine, licensed for administration to chickens, used for this study was protective against *Salmonella* Group B invasion of the spleen, ovary, bursa of Fabricius, ileum and cecum of one to two week old chickens (Hassan and Curtiss, 1994). The same age group chickens challenged with heterologous strains of *Salmonella*, including groups C, D, and E, developed *Salmonella* colonization of the ileum and cecum. Chickens vaccinated at two and four weeks of age and challenged with *Salmonella* Group C also demonstrated intestinal colonization, but at a lower intensity than unvaccinated chickens. *Salmonella* Group C includes many of the *Salmonella* isolates reported from iguanas. In this study, the vaccine was not protective against colonization with a related species, *S.* Typhimurium. If a vaccination program is to be successful at the farm in El Salvador, it must be protective against a range of *Salmonella* serotypes. This presupposes the use of inactivated homologous vaccine, administered parenterally. Immunization with the commercial vaccine may have been ineffective because it was administered to juvenile iguanas at twelve to sixteen weeks of age with an established gastrointestinal microflora that may have impeded the vaccination. The results reported from the field epidemiologic study suggest that obtaining iguanas that are free of *Salmonella* for research and evaluation would be difficult.

In chickens, this vaccine was found to infect multiple tissues and the immunogenicity of *Salmonella* was found to be both dose and genotype dependent

(Hassan et al., 1993). In the iguanas, the vaccine strain was isolated from the lung of 4 (5%) of the vaccinated iguanas, suggesting that the vaccine strain of *Salmonella* did infect the iguanas, but at a low rate. The iguana with the vaccine isolate from group 3 (vaccinated and infected) was negative against the challenge strain of *S.* Typhimurium strain 524. Additional trials to assess the immunogenicity of the vaccine strain in iguanas should be pursued.

There are a number of different factors that may have affected the invasiveness of the vaccine in the iguanas. The vaccine strain of *Salmonella* used in this study does display a diminished resistance to nonspecific host immune responses (Curtiss et al., 1993). Nonspecific immune responses may have cleared the vaccine strain of *Salmonella* prior to invasion and infection of the host cells. The vaccine strain of *Salmonella* would have to survive the gastric acid of the stomach and any non-specific immune responses between the oral cavity and site of infection, which is probably the small intestine or colon. Differences in binding sites at the level of the intestinal enterocytes may have also affected invasion. The microecology of the iguana intestine may have affected the ability of the vaccine strain to colonize and invade host cells. Because the vaccine strain is avirulent, it may have been competitively excluded by other indigenous flora. Further investigation into the pathogenesis of *Salmonella* in the iguana should be pursued. Failure to respond to the vaccine may have been the result of differences in the immunological responses between chickens, which are endotherms, and ectothermic iguanas. The metabolic rate and immune system of an iguana are directly related to establishing an appropriate core body temperature (Klingenberg,

1996). For the vaccine trial, the iguanas were maintained at an appropriate environmental temperature (86-88°F). In chickens, this vaccine (Δ cya andΔ crp) elicits strong humoral, mucosal, and cellular responses against LPS and protein antigens. Immunological responses were not measured in this study because appropriate volumes of blood could not be collected and there is a general lack of knowledge regarding the immune response of the iguana. However, the high degree of infection observed in Group 3 post-vaccination suggests that no protective immune response was generated. The immune system of reptiles is primitive in comparison to higher vertebrates. Lizards are reported to have at least three different immunoglobulins, a high molecular weight Ig-M like immunoglobulin and two lighter weight immunoglobulins (Ambrosius, 1976). Because there are obvious differences in the types of immunoglobulins produced by iguanas and chickens, different responses to a vaccine and infectious strain of *Salmonella* might be expected.

The results of the field epidemiologic study suggest that iguanas are exposed to *Salmonella* at a young age. Although cases of reptile salmonellosis have been reported (Onderka and Finlayson, 1985), the majority of the *Salmonella* positive reptiles show no abnormalities (Jackson and Jackson, 1981; Refai and Rohde, 1968; Zwart, 1962). The iguanas infected with *S.* Typhimurium strain 524 in the infection study and the vaccine study did not develop any clinical signs or gross necropsy changes associated with salmonellosis. Because all of the iguanas used in the studies were previously infected, it is possible that the vaccine and infectious strain of *Salmonella* functioned as indigenous flora.

A shortcoming of the vaccine trial was the failure to infect control animals (group 4). In this study, only 57 % of the control animals in group 4 were infected, as compared to a 90% infection rate reported in the infection study. Nagaraja and Rajashekara (1999) reported a similar (60%) infection rate in poultry used as positive controls for a vaccine trial. The challenge strain of *Salmonella* used for the iguana vaccine trial and the techniques used to propagate and count the organisms were similar between the infection and vaccine studies. One difference between the studies was that iguanas in the vaccine trial were infected 29 days after the completion of the enrofloxacin treatment, whereas iguanas in the infection study were infected 15 days after completion of the enrofloxacin treatment. Changes in the gastrointestinal microflora are expected after the administration of an antibiotic. The number of non-salmonellae microbes isolated from cloacal swabs decreased dramatically immediately after enrofloxacin treatment, but this was not rigorously tested. The difference in time between the completion of the enrofloxacin treatment and the administration of the *S.* Typhimurium strain 524 may have been sufficient for other microbes acquired in the diet to colonize the gastrointestinal tract. Once established, these microbes may have served as an obstacle to infection for the vaccine and challenge strain of *Salmonella*. These results suggest that further research is needed to investigate the role of passive and active immunity in protection against infection.

CHAPTER 6

CONCLUSIONS

The findings of the epidemiologic study in El Salvador confirm that *Salmonella* may infect multiple age classes and both genders of iguanas. Isolation of *Salmonella* from an ovary, internalized yolk sacs from hatchlings, and embryos, suggests that this organism may also be transmitted vertically. The widespread contamination of *Salmonella* on the farm and the direct exposure to the environment, birds, wild lizards, and vermin, precludes the opportunities to create a complete program of disease prevention. Biosecurity measures that may reduce the exposure of naive iguanas to *Salmonella* include routine removal of the fecal material within the pens and daily disinfection of the water basins.

Iguanas are tropical reptiles that require exposure to natural unfiltered sunlight and constant temperatures. Rearing iguanas in sterile indoor facilities, such as those used for laboratory animals in the United States, would be cost prohibitive. The care of captive iguanas is labor intensive. Because labor costs are significantly lower in El Salvador compared to the United States and the market value of the iguana is low ($1.00-2.00/iguana), iguana farming is likely to continue in El Salvador. The findings from this epidemiologic study, in combination with the economic and biological limitations, suggest that eradicating *Salmonella* at the farm level is impossible.

Bayesian methods can be used to estimate the sensitivity and specificity of one or more diagnostic tests in the absence of a gold standard. Limited prior knowledge of the testing parameters may influence the interpretation of results. Although this method

does not provide exact measurements of the test parameters and population prevalence, it does increase our understanding of the tests by providing a range of values incorporating the true value. The PCR assay used in this study was considerably more sensitive than either the ELISA or microbiological culture. The specificity of microbiological culture was higher than the PCR and ELISA assays. Bayesian estimates for microbiological culture sensitivity and specificity were used to calculate the true prevalence of *Salmonella* infection in the iguanas and their environment at the commercial farm in El Salvador, resulting in an increased overall prevalence of infection. Because the PCR assay is both highly sensitive and specific and microbial culture is required for serotyping, parallel testing using both the PCR assay and microbiological culture is recommended. In addition, public health investigators screening iguanas for a specific *Salmonella* serotype in a reptile-associated zoonotic infection should collect multiple colonies for serotyping to improve the probability of identifying the source of the infection.

Salmonella was eliminated from the majority of the iguanas treated with enrofloxacin. Enrofloxacin may be used when *Salmonella* are sensitive to the drug to create a *Salmonella* clearance model. The enrofloxacin clearance models were conducted in sterile environments using food and water that were known to be *Salmonella*-free. The findings of this research indicate that treatment with enrofloxacin can suppress shedding of *Salmonella* for up to 70 days. Veterinarians should not consider using enrofloxacin to eliminate *Salmonella* from apparently normal pet iguanas

because enrofloxacin does not provide long term protection against re-infection with *Salmonella*, which is ubiquitous in the environment.

There are a number of reasons that may have attributed to the failure of the vaccine in this study. However, our limited understanding of the immune response in the iguana and the pathogenesis of *Salmonella* in the iguana only allow one to speculate as to the vaccine failure. Future study to investigate the specific immune responses in the iguana and the pathogenesis of *Salmonella* in the iguana should be pursued.

Pet owners routinely ask veterinarians to examine a pet iguana to characterize its *Salmonella* status. The majority of private practitioners have access only to microbiological culture. This research shows that microbiological culture lacks sensitivity and will lead to the misclassification of infected iguanas. Clients should be informed of the possibility of false negative results and the importance of collecting sequential samples to confirm *Salmonella* status. If *Salmonella* is diagnosed, there is a question of what to do with the result. This research demonstrates that apparently normal iguanas can harbor *Salmonella*. If the iguana does not have specific signs associated with salmonellosis, then treatment is not justified. The use of antimicrobials under appropriate conditions may eliminate *Salmonella*, but there is no evidence of long term protection. If the environment of the iguana is not properly sanitized and disinfected, it is possible that re-infection will occur from the animal's own environment. Contaminated food sources, such as fresh produce, could also serve as a source of infection. Clients should be made aware of the risks of owning iguanas and should be appraised of appropriate husbandry and sanitation procedures. Hand washing

using soap and warm water is an excellent method to eliminate *Salmonella*. Pet owners should be directed to avoid using bathroom basins or kitchen sinks to clean an iguana or any component of its environment. The purchase of a separate washing receptacle, such as a plastic container, is recommended. Dilute bleach solution should be used to clean the environment, and food and water bowls. Substrate should be removed and promptly placed into a waste receptacle.

The high estimated prevalence of *Salmonella* infection reported in the population of iguanas in El Salvador confirms that these animals may pose a health risk to prospective pet owners. Until an appropriate method to eliminate *Salmonella* in iguanas can be found, every iguana should be considered *Salmonella*-positive, a condition that necessitates that strict sanitation procedures be implemented by the pet owner. *Salmonella* are opportunistic pathogens. Thus, immunocompromised individuals are more susceptible to infection and should not keep iguanas as pets. Iguanas should not be recommended as a pet in a household with children under 5 years of age. Determining at what age a child can be trusted with an iguana should be based on the ability to comprehend the importance of hand washing. The majority of the iguana-associated salmonellosis cases reported in infants were the result of indirect exposure to a pet iguana. It is very difficult to maintain a high degree of sterility at all times and a single lapse by a parent could result in the exposure of an infant to *Salmonella*.

Iguanas, like all reptiles, are ectotherms and have special requirements that must be met in captivity. If iguanas are not maintained in appropriate environmental conditions, their metabolism and immune function may be affected. Stress associated

with inappropriate care may affect *Salmonella* shedding. The increase in the prevalence of *Salmonella* in the iguanas on farm 2 as described in section 4.2 may have been associated with the stress of transport. Further research to investigate the effects of environmental stress on iguanas should be conducted. Pet owners should be made aware of the importance of providing an appropriate stress-free environment to reduce the likelihood of shedding *Salmonella*.

Research efforts should focus on increasing an understanding of the cellular, humoral, and nonspecific immune responses in the green iguana. This information may have applications to understanding the pathogenesis of salmonellosis in domestic species and humans. Research should focus on defining the specific pathogenesis of *Salmonella* infection in the iguana, including the interaction with the microecology of the gastrointestinal tract, colonization, invasion, infection, and clearance.

Competitive exclusion products that actively compete with *Salmonella* for colonization sites should be evaluated. There are a number of different commercially available defined and undefined competitive exclusion products developed for commercial chickens that could be evaluated, however differences in the avian and reptile gastrointestinal tract and microecology could affect the outcome. Future studies based on the characterization of the anaerobic bacteria routinely isolated from iguanas would be preferred. Using indigenous microflora, it would be possible to avoid the potential complications associated with avian microbes. Oligosacchardies should also be considered for control of *Salmonella*, as these products may be used to provide a source of nutrition for competing microbes and alteration of the intestinal pH.

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VITA

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DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Mark Anthony Mitchell

Major Field: Veterinary Medical Sciences

Title of Dissertation: Epidemiology of *Salmonella* in the Green Iguana (*Iguana iguana*)

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

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